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RESEARCH ARTICLE

Additive nanomanufacturing of lab-on-a-chip fluorescent peptide nanoparticle arrays for Alzheimer's disease diagnosis

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Abstract

This paper proposes an additive nanomanufacturing approach to fabricate a personalized lab-on-a-chip fluorescent peptide nanoparticles (f-PNPs) array for simultaneous multi-biomarker detection that can be used in Alzheimer's disease (AD) diagnosis. We will discuss optimization techniques for the additive nanomanufacturing process in terms of reliability, yield and manufacturing efficiency. One contribution of this paper lies in utilization of additive nanomanufacturing techniques to fabricate a patient-specific customize-designed lab-on-a-chip device for personalized AD diagnosis, which remains a major challenge for biomedical engineering. Through the integrated bio-design and bio-manufacturing process, doctor's check-up and computer-aided customized design are integrated into the lab-on-a-chip array for patient-specific AD diagnosis. In addition, f-PNPs with targeting moieties for personalized AD biomarkers will be self-assembled onto the customized lab-on-a-chip through the additive nanomanufacturing process, which has not been done before. Another contribution of this research is the personalized lab-on-a-chip f-PNPs array for AD diagnosis utilizing limited human blood. Blood-based AD assessment has been described as "the holy grail" of early AD detection. This research created the computer-aided design, fabrication through additive nanomanufacturing, and validation of the f-PNPs array for AD diagnosis. This is a highly interdisciplinary research contributing to nanotechnology, biomaterials, and biomedical engineering for neurodegenerative disease. The conceptual work is preliminary with intent to introduce novel techniques to the application. Large-scale manufacturing based on the proposed framework requires extensive validation and optimization.

Keywords Nanomanufacturing · Lab-on-a-chip · Fluorescent peptide nanoparticle · Alzheimer's disease · Diagnosis

Introduction

The rapid development of new diagnostic procedures and recent advances in protein chip technologies are driving the development of personalized therapies [1]. For example, this new platform has been utilized for the cancer biomarker discovery and then helped the generation of personalized cancer treatment plans [2]. However, the complexity of these chips also raises a huge challenge and high cost to manufacturing [3–5]. Additive nanomanufacturing is a promising

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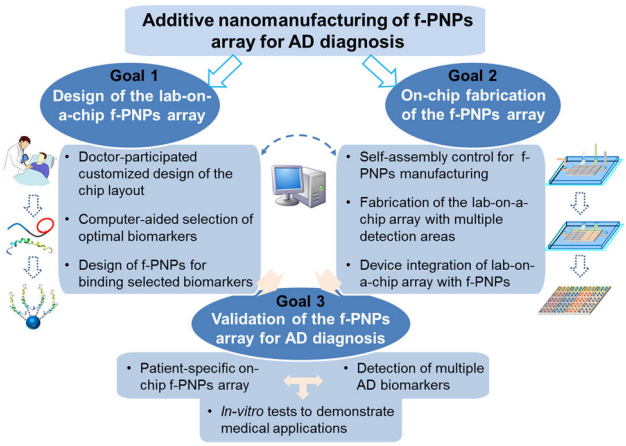

Fig. 1 Flow chart of the overall concept

approach to overcome the concerns by optimizing design and customizing building blocks including DNA, peptides and proteins, to form well-defined architectures associated with desired functionalities and flexibilities [6–10].

As shown in Fig. 1, the goal of this research is to develop an additive nanomanufacturing approach for on-chip fabrication of fluorescent peptide nanoparticles (f-PNPs) array to simultaneously detect multi-biomarkers in personalized Alzheimer's disease (AD) diagnosis. Due to the rapid aging of population, the increasing number of AD patients, characterized by irreversible cognitive decline, memory impairment, and behavioral changes, has become a serious social issue [11]. It has drawn public and scientific communities' attention due to the fact that they are increasing enormously in developed countries and becoming a serious concern for public health. Therefore, AD is emerging as a major concern for society and its diagnosis remains a daunting challenge [12].

Figure 2 illustrates technical details of the additive nanomanufacturing for personalized lab-on-a-chip f-PNPs array for AD diagnosis using human blood sample. *First*, data including patient family and personal medical history along with cognitive assessments will be collected during doctor's check-up, and processed by computer. Through interactions with the medical doctor, computer algorithm will (1) generate a list of potential biomarkers from existing library, (2) propose appropriate peptide sequences forming f-PNPs to detect the biomarkers, and (3) recommend the overall chip layout for the medical doctor to make final decision. After doctor's final decision, the personalized lab-on-a-chip design will be sent to fabrication lab to in situ self-assemble the f-PNPs array that can detect the selected AD biomarkers. The f-PNPs will be self-assembled using specific peptides with desired sequence. Selection of the biomarkers will determine the array fabrication and arrangements of the f-PNPs. *After chip fabrication*, human serum will flow through the f-PNPs array that has the ability to recognize and bind with multiple AD biomarkers. *Finally*, fluorescence intensity and nanomorphology of the f-PNPs array will be screened and processed using a computer controlled optical detection and imaging platform. The concentration and aggregation stages of the AD biomarkers can be detected through monitoring the changes of fluorescence signal and nanomorphology. Using a predictive model published in Science Advance [13], recommendation to patient's AD diagnosis will be generated. The bottleneck for the above process lies in rapid, accurate, and cost-effective manufacturing of the customized lab-on-a-chip design, which is the focus for this research. Specifically, the focus of this research is on additive nanomanufacturing of the on-chip self-assembly process of the monolayer f-PNPs arrays, which can provide sensitive and diagnostic biomarker-specific information for AD diagnosis. The manufacturing principles learned through this research can be applied to various additive nanomanufacturing processes for lab-on-a-chip biological and biomedical applications.

Backgrounds

Currently, no single biomarker can be used to reliably diagnose AD [14, 15]. There is an urgent need for AD diagnosis using combined biomarkers. Lab-on-a-chip technique is a highly promising candidate for this purpose, due to its high-throughput and sensitive screening capabilities [16]. It has been shown that combined analysis of multiple biomarkers can provide patient-specific signatures distinguishing AD with other dementias and be used to determine AD stages [17]. Additionally, among different human fluid resources, blood-based AD assessment has been regarded as "the holy grail" for early AD detection owing to the ease and low cost of acquisition, the large number of analytes contained within it, and its suitability for preclinical population-based screens. However, selection of proper biomarkers from the long list of potential AD biomarkers inside the blood is challenging for efficient and cost-effective AD diagnosis [18]. By integrating individual patient's medical history and cognitive assessments, doctors are capable to narrow down potential biomarker candidates and determine a patient-specific selection. Lab-on-a-chip technology provides a tremendous, but unproven potential to improve diagnosis efficiency [19]. Chip-based technologies have several advantages over conventional bio-analysis systems, because (i) they enable rapid analysis of a large number of samples in a single experiment, (ii) there is reagent economy, and (iii) the signal-to-noise ratio exhibited by the chips is much better than that observed for conventional assays [20–22]. On the other hand, the use of peptides for constructing selective functional nanostructures has tremendous applications as functional components for lab-on-a-chip devices toward specific demands [23]. Peptides

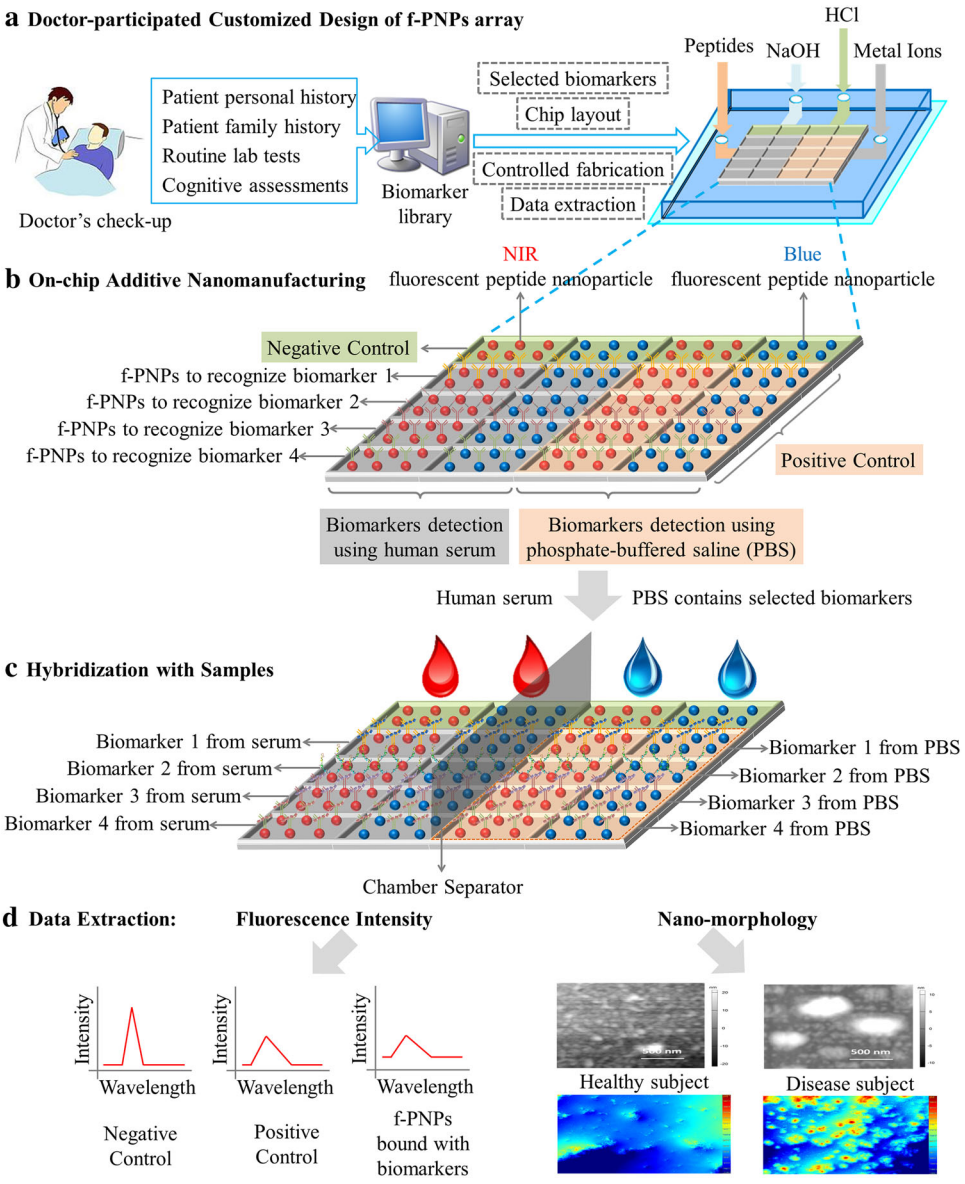

Fig. 2 A schematic drawing of the f-PNPs array for AD diagnosis. **a** The overall design of the lab-on-a-chip f-PNPs array. **b** Blue and NIR f-PNPs array with separate functional areas to detect multi-biomarkers. **c** After introducing serum and PBS (*positive control, containing selected biomarkers*), the f-PNPs array will specifically bind with biomarkers. **d** Fluorescence intensity and nanomorphology changes of the f-PNPs can reflect the concentration and aggregation of biomarkers

have demonstrated their exceptional benefits in developing self-assembled nanostructures and devices with expected functionalities [24]. Our previous research has demonstrated the feasibility of using fluorescent dipeptide nanoparticles as functional nanoprobes for targeted cell imaging and real-time monitoring of drug release [25]. Furthermore, lab-on-a-chip technology has significant advantages in regulating microenvironment and integrating with automation systems that are critically needed for controlling peptide self-assembly to achieve specific sequences and nanostructures. Based on doctor's check-up, the f-PNPs arrays will be designed with targeting sequences to bind with specific biomarkers and fabricated through additive nanomanufacturing. The barriers that the on-chip self-assembled f-PNPs need to overcome include cost, speed, dynamic range and personalized manufacturing based on individual patient's condition, for which additive nanomanufacturing provides the best options.

Results

Bio-design

Computer-aided design for the lab-on-a-chip f-PNPs array

The f-PNPs arrays have been designed based on doctor's check-up, patient's medical history and family history. Computer-aided design will be utilized to generate the layout of the lab-on-a-chip array and the selection of biomarkers from a library based on interactive exchange of informa-

tion with the medical doctor. The goal is to select a small amount of biomarkers to be put into the chip and used for the f-PNPs array detection. The peptide sequences of the f-PNPs array will be designed according to optimized self-assembly pattern and effective targeting capability to the selected biomarkers.

(a) Generation of the doctor-participated design requirements for the chip layout.

In the additive manufacturing process, computers will be used to generate the doctor-participated design requirements for the chip layout, including inlets for peptide introduction, fabrication procedures for both the chip and the f-PNPs, and the number of detection chambers on the array. The design will be determined by the health conditions of individual patient and the specific conditions from their doctors. Patient's medical history and cognitive assessment results will be collected in the meantime and analyzed by the computer that can be used to assist with AD biomarker selection as well. *First*, the chip layout will be determined including the design of each layer according to the microchannels connecting inlets and outlets, the microvalves for microenvironment control, and the size of detection chambers for the f-PNPs array. *Then*, the requirements for biomarker selection will be generated based on the doctor's recommendation. It includes the type and number of the biomarkers that can be used to diagnose the patient's disease. *Finally*, the fabrication procedures for the lab-on-a-chip device will be determined, including device fabrication steps, self-assembly procedures and control approaches. The overall additive nanomanufacturing process will be monitored and controlled using computers.

(b) Computer-aided selection of AD biomarkers.

Based on patient's personal medical and family information, and doctor's recommendation, limited personalized biomarkers will be selected from the biomarker library using a computer and through interaction with their medical doctors. These patient-dependent biomarkers are expected to have minimal types of selection with maximal detection capability. As demonstrated in the preliminary studies, the f-PNPs array can be modified to specifically recognize and detect amyloid-beta polypeptides (Aβ) and other protein biomarkers through measuring changes of nanomorphology and fluorescence intensity. The selected biomarkers will determine the design of the f-PNPs. Patient's cognitive assessments will also contribute to biomarker selection. According to the potential symptoms of particular dementia and progression stages, the selection will be various. Computer will select a group of biomarkers from the biomarker library of this particular dementia type and disease stage. To reduce the diagnosis cost and obtain as much as possible information for disease diagnosis, the selection process can be further optimized. The selected biomarkers will be analyzed one by one in terms of the detection mechanisms and disease correlations. The computer will evaluate the correlation coefficients for each biomarker to determine the important ones. After optimizing the biomarker selection, the arrangement of the array layout can be determined, including the biomarkers targeted on each row and column. The final detection patterns of the array for different disease conditions will then be established.

(c) Design of the f-PNPs for targeted binding with Aβ polypeptides and AD protein biomarkers.

The f-PNPs will be used to detect different biomarkers in patient blood through monitoring the changes of fluorescence intensity and nanomorphology. One potential problem for obtaining the fluorescence signal is that the fluorescent amino acids are relatively rare in proteins [26]. Another potential problem is that the fluorescence signal from amino acids is situated in the ultraviolent range, which is pernicious, invisible and difficult to be imaged. We have made breakthroughs on designing and fabricating visible fluorescent dipeptide nanoparticles (emission wavelength: 423 nm), which are biocompatible, biodegradable and photostable [25]. Meanwhile, peptides may have the tendency to cluster, generating sophisticated architectures spontaneously via self-assembly without harmful and expensive catalytic reaction. The diversity of the amino acid candidates for the sequence design allows the feasibility of developing multifunctionalized nanomaterials [27, 28]. In this research, both the fluorescence intensity and nanomorphological changes from the f-PNPs array can be used for distinguishing the AD biomarkers.

Design of the recognition sequence As several peptides KLVFF (Lys-Leu-Val-Phe-Phe), WF (Trp-Phe), LVFFAE (Lys-Val-Phe-Phe-Ala-Glu), KVLFFAE (Lys-Val-Leu-Phe-Phe-Ala-Glu), LVFFARK (Lys-Val-Phe-Phe-Ala-Arg-Lys) have been discovered to selectively bind with Aβ polypeptides [29–32], Fig. 3 shows the library of the self-assembly and recognition of peptide sequences. To demonstrate the feasibility, KLVFF will be synthesized and incorporated into the proposed peptides. Specifically, the sequence KLVFF (corresponding to Aβ [16–20]) will bind with full-length Aβ polypeptides [31]. In addition, the recognition sequence needs to be protected prior to the self-assembly. To achieve this goal, cysteine will be embedded into the recognition sequence on both C-terminal and N-terminal. Disulfide bond will be formed under gentle stirring in 8 M guanidine hydrochloride [33]. This cyclization process will reduce the reaction activity and protect the recognition

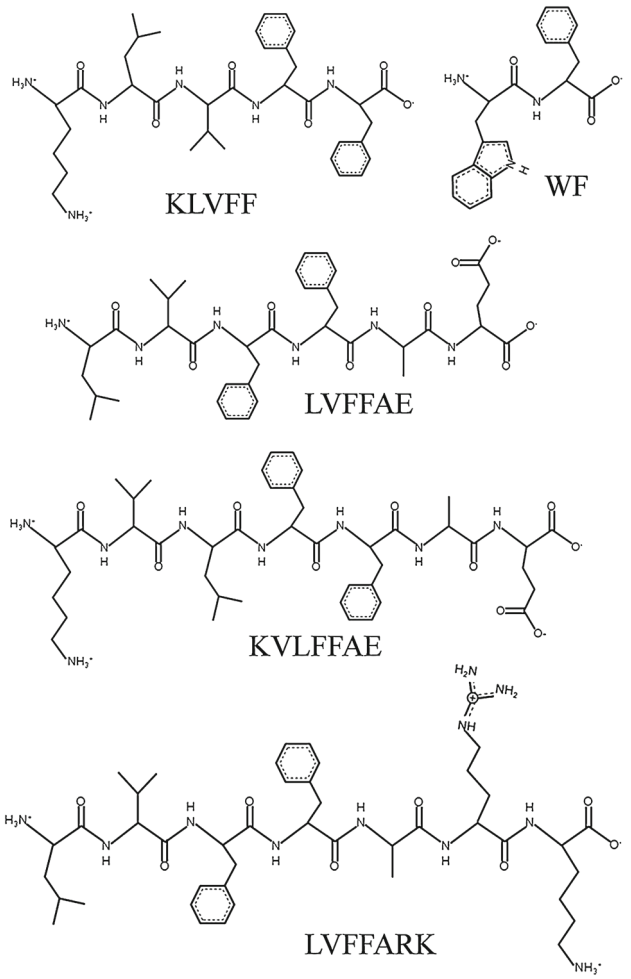

Fig. 3 Structures of the self-assembly and recognition peptide sequences

sequence during self-assembly process. After self-assembly, the disulfide bonds will break down with 3 μM tris(2-carboxyethyl)phosphine at slightly alkaline pH to be able to recognize the Aβ polypeptides [34].

Design of the linker sequence Given direct fusion of self-assembly and recognition sequence without flexible linker may lead to undesirable outcomes, including inefficient self-assembly and malfunction of the recognition sequence. Therefore, the design and incorporation of a linker sequence is important for on-chip self-assembly and biomarker detections. A flexible liner linker will be imparted into the peptide design to connect the two respective domains functionalized toward recognition and self-assembly [35]. To find out the best linker sequence for both self-assembly and recognition, flexible linker $(AA)_n$ ($n = 1, 2, 3, 4$) will be embedded and optimized to obtain the best outcomes.

Design of the self-assembly sequence Based on our previous research experience, tryptophan-phenylalanine (WF) dipeptide is chosen as effective self-assembly sequence to induce the formation of organized nanostructure under controlled microenvironments. The overall design of the peptide would be [C-recognition sequence-C]-[linker sequence]-[self-assembly sequence]. Several proteomic resources will be exploited to support and direct the design of the peptides. The hierarchical self-assembly progress of the putative nanostructures and the distribution of corresponding recognition domains will be predicted. In particular, a series of theoretical calculations associated with proteomic tools, including the establishment of the structure homology model, the prediction of the helical content of peptides, secondary structure prediction, theoretical PL (isoelectric point) and MW (molecular weight) computation, identification of functional motifs, hydrophobic cluster analysis, and many others will be performed to predict the architecture and physicochemical properties of the designed peptide nanostructures prior to the fabrication.

The f-PNPs array for binding with tau protein As there is no peptide to specifically bind with tau protein, the peptides are firstly designed for effective self-assembly into the fluorescent nanostructure. WF or cyclo[-(AEAW)$_2$−] as self-assembly sequences will be synthesized and self-assemble into the f-PNPs array on the chip. The f-PNPs will then be modified with anti-tau antibodies to detect tau proteins on-chip. The f-PNPs/antibody conjugates will be synthesized through conjugation of carboxyl terminated peptide nanoparticles and amine terminated anti-tau antibodies using catalysts 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). To activate carboxyl groups, f-PNPs will be treated with EDC and NHS for reaction of ~ 25 min. The mixture will be centrifuged at 5000 rpm for 30 min and dissolved in distilled water. After activation of carboxyl groups from f-PNPs, the anti-tau antibody will be dropped onto the activated f-PNPs arrays under room temperature overnight. Extra unbounded antibodies will be washed out with phosphate-buffered saline.

Computer-aided selection of biomarkers for personalized AD diagnosis

As illustrated in Fig. 2, the design and control for the f-PNP self-assembly are keys to realizing the computer–doctor interactions for customized lab-on-a-chip fabrication. In general, multiple biomarkers are needed to improve the sensitivity and accuracy of AD diagnosis. Based on the broadly accepted database of NINDS and ADNI, as well as resources from private companies, such as SOMAscan® for biomarker discovery, we have included more than one thousand AD biomarkers as the library. Next, choose the right biomarkers

is becoming a major concern because too many biomarkers are costly. To overcome the concern, we have tested the idea of doctor-participated biomarker selection to reduce the numbers based on cognitive assessments and medical history of individual patients. It was shown that SAGE is an improved cognitive assessment for screening individuals with mild cognitive impairments, subjective memory complaints, or other early dementia [36]. We recruited patients using their SAGE scores to classify them into different AD stages. The selection of biomarkers is altered for different stages. By analyzing cognitive assessment scores at multiple time points, the changes of scores provide information about the potential dementia type for distinguishing AD and other memory disorders. Multiple computational approaches are available to calculate the correlation coefficient of each biomarker with the disease conditions to determine the priority of each biomarker. To demonstrate principles of the design, fabrication and validation of f-PNPs array, we decide to use the most common biomarkers amyloid-beta (Aβ), tau, their positive and negative controls in this preliminary study.

Computer-aided design of the fluorescent peptide nanoparticles with targeted binding sites for AD diagnosis

As shown in Fig. 4, *first*, the candidate peptides are designed for recognition and binding with the Aβ polypeptides or acting as the building blocks to self-assemble into the fluorescent nanostructures. Specifically, the peptide sequence labeled with red color will bind with Aβ polypeptides—a major AD biomarker. The peptide sequence labeled with blue color in Fig. 4 will self-assemble into nanostructures. After the peptide synthesis, the cyclization process between two cysteine amino acids will be introduced to reduce the reaction activity and protect the recognition sequence during the self-assembly process. In addition, cysteine will be embedded into the recognition sequence on both C-terminal and N-terminal in order to form cyclization to protect the recognition sequence during the self-assembly. *After that*, self-assembly parameters (*pH, temperature, and ion concentrations*) will be controlled for peptide on-chip self-assembly. *Finally,* disulfide bond of the self-assembled f-PNPs will break down under certain conditions to recognize the Aβ polypeptides. Similarly, f-PNPs targeting other biomarkers will be on-chip self-assembled to form single-layer arrays. The above step will ensure fabrication of the on-chip arrayed f-PNPs for sensitive detection of multi-biomarkers.

Bio-manufacturing

Fabrication of the lab-on-a-chip f-PNPs array

We utilize the idea of computer-centered additive nanomanufacturing to produce the lab-on-a-chip f-PNPs array. The

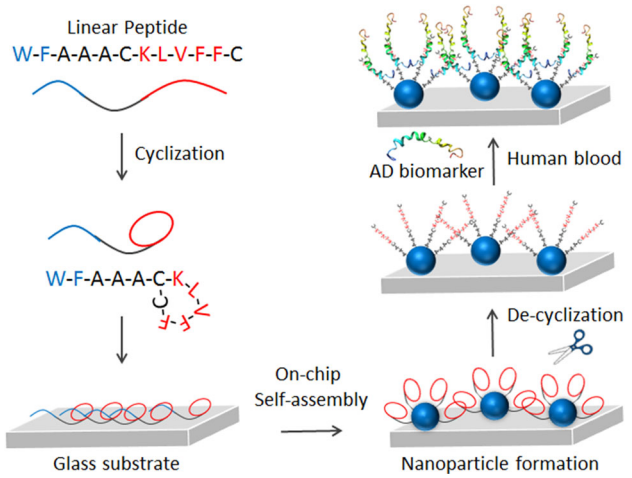

Fig. 4 Self-assembly of peptide sequence-dependent biomarker detection. First, WFAAACKLVFFC peptide will be synthesized and purified. The cyclization process between two cysteines will be introduced to reduce the reaction activity and protect the recognition sequence. After the self-assembly, the disulfide bond will break down to bind with biomarkers. The aggregation of biomarkers can be detected by measuring fluorescence intensity and nanomorphology changes

steps, including peptide self-assembly and lab-on-a-chip array fabrication, will be controlled by the computer. Specifically, the microenvironment will be controlled through different channels and valves of the chip. The array will be fabricated based on the patient-specific selection of biomarkers and the detection signals.

(a) Control the self-assembly procedures of the f-PNPs.

The designed peptides will be first synthesized, cyclized and purified for the on-chip self-assembly. In our previous studies, considerable progress has been made in the tunable synthesis of self-assembled peptide nanoparticles using multiple strategies, including phase equilibrium, pH-driven, and pH-sensitive approaches [37]. In this research, the peptide sequence will be utilized for three functions: ordered self-assembly, fluorescence property and recognition to particular AD biomarkers inside human serum. The on-chip self-assembly process will be controlled through the multiple microchannels on the chip. One of the fundamental aspects of the self-assembly control on nanoparticles is the environmental parameters. We can utilize additive nanomanufacturing procedure and apply the aggregates of peptides as building blocks. Inside the chip, self-assembly process will be controlled to achieve desired structures and functionalities.

Computers will be used to generate a fully controlled self-assembly environment to produce the peptide nanoparticles on the chip. Here, multiple microchannels and microvalves will be fabricated on the chip to control the peptides, sol-

vents, metal ions, pH and concentration. Temperature and pH value of the peptide microenvironment will be precisely controlled to form organized nanostructure. The pH will be adjusted to 11 and the temperature will be set to 75 °C for 30 min. Specifically, the microchannels inside the chip are connected together so as to achieve a desired function (mix, pump, redirect or control chemical reactions). This network of microchannels is connected to the outside by inputs and outputs pierced through the chip, as an interface between the macro- and microworld. Through these holes, the chemical reagents in solution are injected and removed from the lab-on-a-chip. On the other hand, functional microvalves will be utilized for conducting flow control and manipulation tasks. To fully control the solution, normally closed microvalve will be selected [38]. These valves are pneumatic microvalves based on the deformation of thin Polydimethylsiloxane (PDMS) layers. A pneumatic layer will be enabled to control the deformation of the PDMS layer, hence enabling or disabling fluid flow through the holed layer. Thus, with the syringe pumps for solution control and the pneumatic pumps for valve control, computer programs will be developed to customize the self-assembly according to different peptide sequences and binding sites. Through optimization, uniform spherical f-PNPs will be formed and arrayed on the modified chip. Concentration of the self-assembled peptides, stirring speed and reaction time will be optimized to make sure the formation of single-layer f-PNPs, which will be verified through the height information of synthesized f-PNPs using AFM. The self-assembled f-PNPs can specifically bind the Aβ polypeptides and tau proteins, as well as other potential biomarkers, based on the changes of nanomorphology and fluorescence intensity, characterized with AFM, DHM [39] and a solid-state fluorescence spectrometer.

(b) Fabrication of the lab-on-a-chip array with multiple detection areas.

The chip with multiple reaction chambers is prepared by transferring the desired pattern into one PDMS substrate via soft photolithography techniques. The chip layout will be provided by the computer-aided design based on doctor's check-up and patient's requirements. Specifically, the detection areas will be designed following the biomarker selection. *Negative control* area will be composed of the f-PNPs without specific binding ability. *Positive control* area will be the duplicate of the blood-based detection area, which will be isolated by the chamber separator. Specifically, once the design of chip array is ready, it will be sent to direct the photomask fabrication that has the transferred pattern of the array design. Based on the mask, the mold of the array will be made by photolithography. This is the step when the drawings of the microchannels on the photomask are transformed into real microchannels (the mold). Microchannels and chambers are "sculpted" on the mold, resulting in replicas that will enable carving the patterns into the PDMS. (1) Resin is spread on a silicon wafer with the desired thickness (which determines the height of the channel). (2) The resin, here we will use SU-8, protected by the mask on which the patterns are drawn, is partially exposed to UV light. Thus, only the parts representing the channels are exposed to UV light and cured, the other parts of the mold being protected by the opaque areas of the mask. (3) The mold is developed in a solvent that etches areas of resin that were not exposed to UV light. (4) We obtain a chip mold with a resin replica of the patterns that were present on the photomask (future microchannels are the remained resin on the mold). The height of the channels is determined by the thickness of the original resin. The molding step allows mass-producing chips from a mold. (5) A mixture of PDMS (liquid) and crosslinking agent (to harden the PDMS) is poured into the mold and placed in a furnace. Once the PDMS is hardened, it can be taken off the mold. (6) A replica of the channels on the PDMS block is obtained. To allow the injection of fluids for future experiments, the inputs and outputs of the chips are punched with a needle or a punch of the size of future outer tubes. (7) Finally, the face of the PDMS block with microchannels and the glass slide surface are treated with plasma, in order to bond and close the chip. With the flow and gas control system, the control of the microenvironment inside can be performed.

(c) Device integration of the lab-on-a-chip array with functional f-PNPs.

The f-PNPs array will be built on the chip. The f-PNPs with recognition sequence will be seeded on the surface for detecting particular biomarkers. First, the moistures will be removed under lyophilization after self-assembly. Other than recognition peptide sequence, anti-tau antibodies will also be conjugated with WF self-assembled nanoparticles. We will investigate the coating efficiency and results based on the different substrate including patterns, roughness, and elastic modulus. Afterward, AFM, DHM, solid-state fluorescence spectrophotometer and confocal fluorescence microscopy will be applied to study the interactions between f-PNPs and biomarkers, and to find out the optimized peptide sequence with recognition ability to biomarkers.

(d) Self-assembly of visible fluorescent peptide nanoparticles for targeted tracking.

Inspired by the π–π stacking induced red-shift from green fluorescent protein (GFP) to yellow fluorescent protein (YFP) through genetic mutation, and the Zn(II) mediated structure rigidification that enhances the fluorescence intensity of the GFP mutant (BFPms1), a tryptophan-phenylalanine WF dipeptide was developed to demonstrate fluorescent

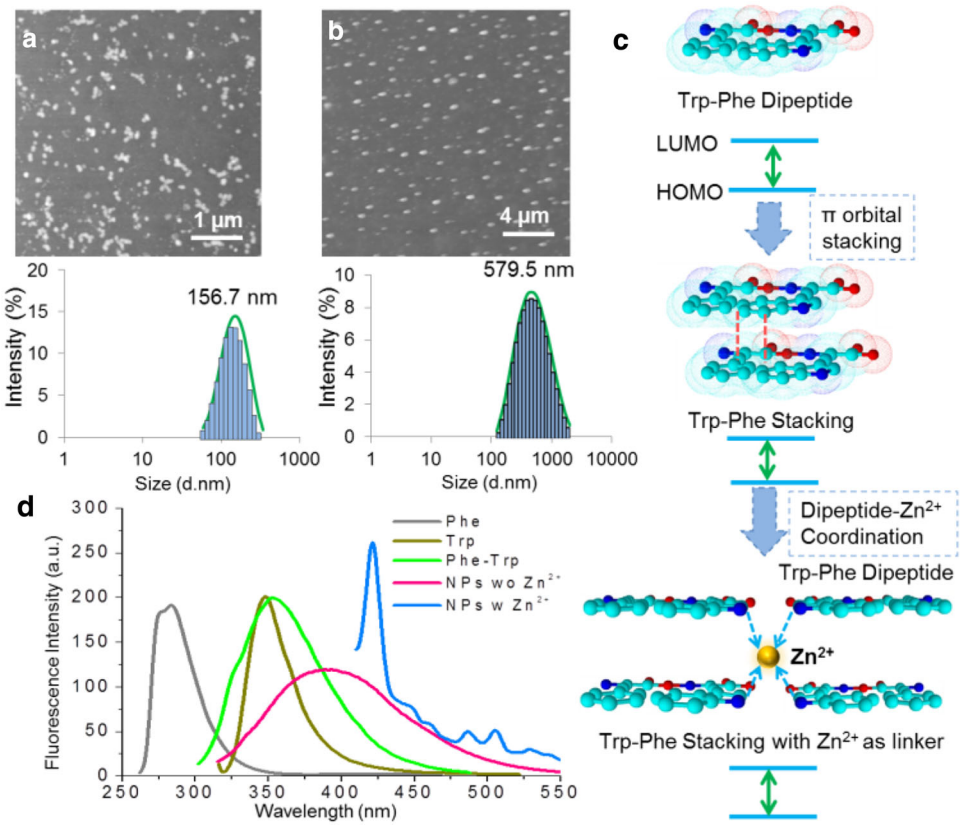

Fig. 5 Fluorescence enhancement and red-shift within the f-PNPs. **a**, **b** Dipeptide nanoparticles with and without Zn^{2+} coordination. **c** Self-assembly of the nanoparticles and red-shift of fluorescence. **d** Fluorescence emission spectra of various f-PNPs. After π–π interaction and Zn^{2+} coordination, fluorescence emission of f-PNPs was red-shifted to 423 nm in the visible range. By designing the sequence of f-PNPs, biomarkers can be selectively and sensitively detected by monitoring the change of fluorescence intensity and nanomorphology. Reprinted from Ref. [23] with permission from Nature Publishing Group

enhancement and red-shift principle [25]. WF dipeptides were prepared at a concentration of 5 mg/mL. Peptide nanoparticles were obtained by reaction of WF dipeptides with $ZnCl_2$ in a mixture of aqueous NaOH and methanol. The pH was adjusted to 11 with HCl. The reactants were heated to 75 °C for 30 min. The intermolecular π–π interactions and Zn coordination are the driving force of peptide self-assembly as shown in Fig. 5c. Once the π–π stacking orients nanoparticles formation, the fluorescent emission shifts to longer wavelength. Besides, fluorescence red-shift is a result of the reduced HOMO–LUMO energy gap after the dipeptide self-assembly driven by the π–π stacking [40]. Through the Zn(II) coordination, fluorescence intensity increased consistently in comparison with that of dipeptide nanoparticles self-assembled without Zn(II) coordination. AFM image in Fig. 5a, b shows that majority of dipeptide nanoparticles with Zn(II) remained spherical shape, but with a smaller size about 160 nm in diameter. As shown in Fig. 5d, fluorescence emission wavelength of the dipeptide nanoparticles was around 390 nm, which was longer than that of Trp-Phe dipeptide monomer (352 nm), tryptophan (348 nm), or phenylalanine (281 nm). Eventually, fluorescence emission band of dipeptide nanoparticles with Zn^{2+} was red-shifted to 423 nm, which can be observed in the visible light range.

Fabrication and integration of the prototype lab-on-a-chip device to control the microenvironment on the chip

The prototype lab-on-a-chip array is fabricated using PDMS and soft photolithography approaches [41]. As shown in Fig. 6, the device has multiple inlets to control the microenvironment for on-chip self-assembly and biomarker detection, including the solvent, peptide type, concentration, temperature and pH. *First*, arrays with multiple detection areas are prepared. Each area of the glass substrate is modified on a surface according to different sequence design. *After that,* the arrays are covered by the PDMS chip, forming a chamber and two side channels with 6 holes as inlets and outlets (Fig. 6a). Before injecting the solution containing biomarkers to the chamber, selective peptides are introduced through side channels into the array chamber for self-assembly to form single-layer f-PNPs array. In this prototype chip, two types of peptide solutions were simultaneously injected into the chambers for self-assembly (Fig. 6b). A stock solution of WFAAACKLVFFC peptides was imported into the assembly area to initiate the nanoparticle formation. Glass chips with reactants were placed in the oven with gradient heating to 75 °C. Afterward, the solution on the chip was cooled down and kept at room temperature overnight. By controlling the peptide supply of each inlet, the peptide nanoparticles with

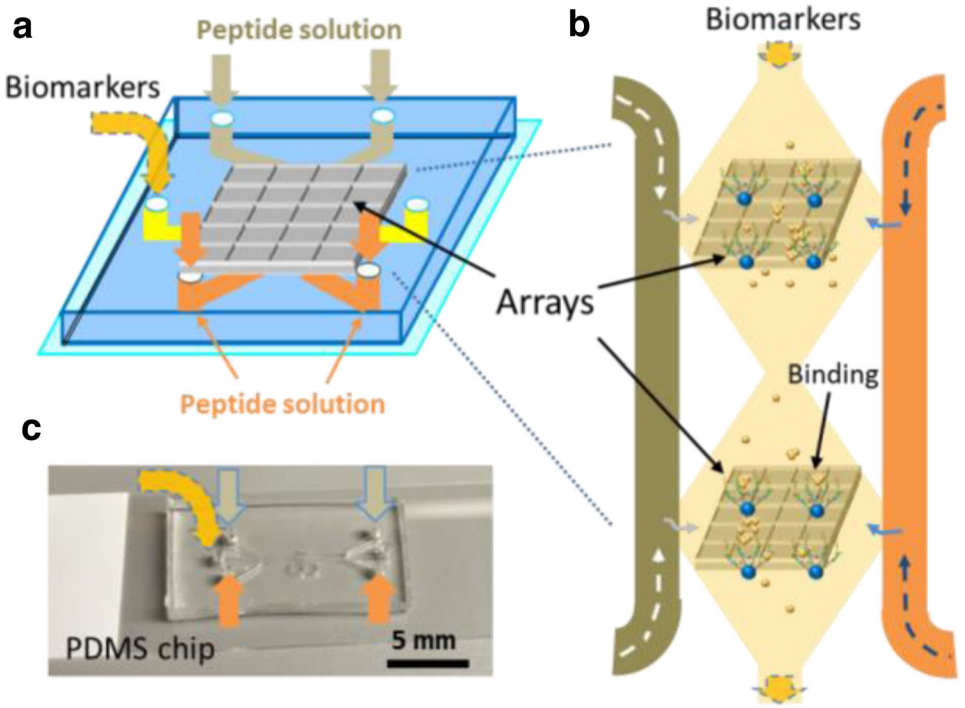

Fig. 6 A prototype lab-on-a-chip device for on-chip self-assembly of the f-PNPs array. **a** The device design for on-chip self-assembly of f-PNPs array. **b** Top view of the device design with two array chambers for self-assembly control and array fabrication. **c** A photograph of the device. On-chip self-assembly of the f-PNPs array can be performed in the prototype device

desired sequences can be obtained in different array areas that can be used for detecting AD biomarkers.

Sample solutions containing AD biomarkers are then introduced from the center inlet and flow across the whole array (Fig. 6b). Based on different binding sites of the f-PNPs array, biomarkers will be trapped into different array areas. As mentioned above, the fluorescence properties will be utilized to distinguish the biomarkers and generate the disease associated molecular information for the subjects. In addition, several arrays are lined in series in the device to evaluate the concentration effects to the peptide self-assembly. Figure 6c shows how the device may be used.

On-chip self-assembly of designed peptides for sensing Aβ polypeptide and ICAM-1 protein

Fluorescence intensity and nanomorphology changes of the f-PNPs array treated with serum from healthy human and AD patients are shown in Fig. 7. The human serum samples are obtained from Buckeye Biospecimen Repository. Serum was controlled to flow through the f-PNPs array for Aβ detection. Upon binding with Aβ polypeptides, the nanomorphology and fluorescence intensity of the f-PNPs array would be different. As shown in Fig. 7a, although clear intensity drop was observed from the f-PNPs array with serum from healthy human, significant intensity drop was observed from the f-PNPs array with serum from AD patient. In addition, clear aggregations were observed from the f-PNPs array with serum from healthy human in Fig. 7b, c. The f-PNPs array treated with serum from AD patient demonstrated severe aggregations in Fig. 7d, e. The Aβ aggregation in serum could be detected by the change of fluorescence intensity and nanomorphology.

In addition, we tested the f-PNPs array for detecting other potential protein biomarkers for AD diagnosis. Here, the ICAM-1 protein was utilized as an example. *First*, synthesized WF peptides were self-assembled to form single-layer f-PNPs array on the glass surface. *Then*, the f-PNPs array will be modified with anti-ICAM-1 antibody. The serum was controlled to flow through the f-PNPs array for ICAM-1 detection. As shown in Fig. 8a, while clear intensity drop was observed from the f-PNPs array with serum from healthy human, the array with serum from AD patient demonstrated significant intensity drop due to much higher concentration of ICAM-1. Mild and severe aggregations were discovered from f-PNPs treated with serum from healthy human and AD patient in Fig. 8b–e. The fluorescence intensity drop was likely due to the massive conjugation between f-PNPs and target protein ICAM-1. The ICAM-1 aggregation in serum could be detected by the change of fluorescence intensity and nanomorphology.

Validation of the lab-on-a-chip f-PNPs array for detecting AD protein biomarkers

Based on above goals, doctor-participated and customized lab-on-a-chip f-PNPs array is obtained for validation. Selected AD biomarkers inside the serum will be detected and analyzed by the f-PNPs array, based on the changes of fluorescence intensity and nanomorphology.

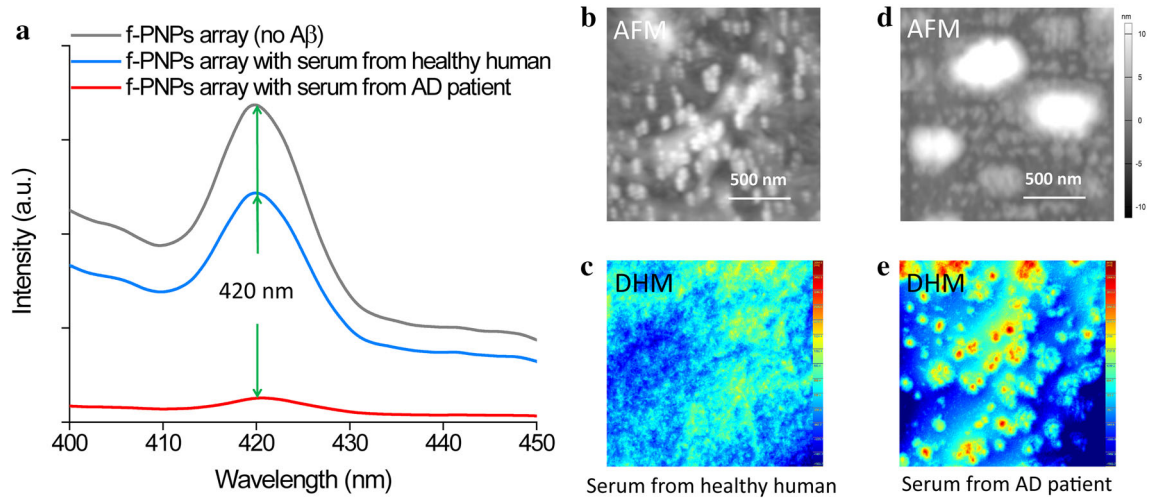

Fig. 7 Fluorescence intensity and nanomorphology changes of the f-PNPs array treated with serum from healthy human and AD patients. **a** On glass chip, solid-state fluorescence emission spectra of the f-PNPs array with analytes. Clear intensity drop was observed from the f-PNPs array with serum from healthy human. Furthermore, the f-PNPs array with serum from AD patient demonstrated significant intensity drop. AFM and DHM images of the f-PNPs array with serum from healthy human **b**, **c**, serum from AD patient **d**, **e**. Clear aggregations were observed from the f-PNPs array with serum from healthy human. The f-PNPs array treated with serum from AD patient demonstrated severe aggregations. The Aβ aggregation in serum could be detected by the change of fluorescence intensity and nanomorphology

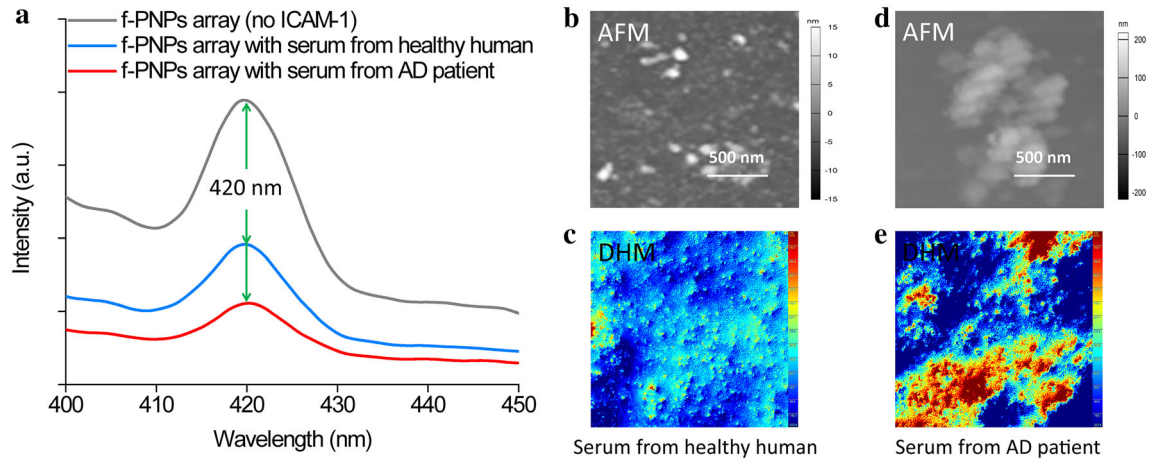

Fig. 8 Fluorescence intensity and nanomorphology changes of the f-PNPs array treated with serum from healthy human and AD patients. **a** On glass chip, solid-state fluorescence emission spectra of the f-PNPs array with analytes. The f-PNPs array with serum from AD patient demonstrated significant intensity drop due to much higher concentration of ICAM-1. AFM and DHM images of the f-PNPs array with serum from healthy human **b**, **c**, serum from AD patient **d**, **e**. Clear aggregations were observed from the f-PNPs array with serum from healthy human. The f-PNPs array treated with serum from AD patient demonstrated severe aggregations. The ICAM-1 aggregation in serum could be detected by the change of fluorescence intensity and nanomorphology

Implementation of the patient-specific on-chip f-PNPs array

We first integrate the data of a particular patient into the additive nanomanufacturing to obtain the patient-specific on-chip f-PNPs array. Our research group through collaboration with an expert AD clinician has made significant progress in utilizing f-PNPs arrays to detect and analyze multiple AD biomarkers as shown in the preliminary data. Based on various peptide nanoparticles produced from the lab-on-chip device, we can integrate selected biomarkers that are specific for this patient. The lab-on-chip device will have special patterned chambers and channels in which the patient-specific f-PNPs are arrayed.

Specifically, this f-PNPs array will be integrated with multiple detection areas for different AD biomarkers as shown in Fig. 2b. The blue and NIR f-PNPs arrayed microfluidic chip with separate functional areas to detect multi-biomarkers. After treated with the blood sample and PBS containing biomarkers (positive control), f-PNPs arrays will recognize and target AD biomarkers. Upon binding with biomarkers

(Fig. 2c), the fluorescence intensity and nanomorphology of f-PNPs arrays will be different (Fig. 2d). Fluorescence intensity and nanomorphology changes will be applied as signatures of specific targets. Solid-state fluorescence spectroscopy, DHM and AFM will be applied to screen both nanomorphology and fluorescence intensity, which reflect the concentration and aggregation stages of biomarkers.

Detection of multiple AD biomarkers using the f-PNPs array

This patient-specific on-chip f-PNPs array has targeted binding sites for multiple protein biomarkers. Specifically, Aβ polypeptides, tau protein, biomarkers of inflammatory and some other disease-related biomarkers will all be incorporated into this validation. The advantages of the high-throughput assay from the array provide a feasible tool to detect various proteome biomarkers inside serum and reveal differences between healthy people and AD patients. The on-chip array contains multiple positions coated with modified single-layer f-PNPs with various targeting sequences.

As the fluorescence intensity of the f-PNPs arrays will change after binding with Aβ polypeptides and tau proteins, we can determine the aggregation status and concentration of biomarkers. A two-tailed unpaired *t* test will be applied to compare concentrations of different biomarkers. The obtained concentrations will be used later to correlate with the fluorescence intensity and nanomorphology change from the single-layer arrayed f-PNPs. Then, the quantification of Aβ and tau, as well as other AD biomarkers, will be conducted. Here, how much changes of fluorescence intensity after binding with Aβ polypeptides and tau proteins will provide quantitative analysis data. We will use the changes obtained from fluorescence emission spectra to quantitatively analyze the selected biomarkers from human serum. In addition, nanomorphology change will be characterized by AFM and DHM. Aggregation will be discovered upon binding of the f-PNPs arrays with biomarkers. The aggregations of AD biomarkers and f-PNPs will block the electrostatic interaction and charge transfer of f-PNPs and induce the fluorescence intensity drop. Thus, we can distinguish the type of target biomarkers, their concentration and aggregation status based on the changes of nanomorphology and fluorescence intensity, characterized with AFM and solid-state fluorescence spectrometer which can be applied for AD diagnosis.

In vitro tests to demonstrate the personalized medical applications

Human serum will be dropped into the device as an in vitro test. Due to the complex biological components of human serum, isolation and purification of the protein components containing selected biomarkers are regarded as important steps. The density gradient centrifugation will be used to separate the protein contents inside the serum to different layers. Then, we will decrease the potential protein types within a small range. The key parameters of this isolation include the centrifugation speed and time, as well as the solvent pH and temperature. *First*, the solvent pH and temperature will be set at 7.4 and 4 °C, to prevent the damages to the proteins. *Second*, we will set centrifugation at 37,500 rpm (RCFav 132,000 g) for 16 h and 30 min at 4 °C with an acceleration profile of 7 and deceleration profile of 7. In order to optimize the procedures, 2 sets with the same gradients will be prepared to compare with each other, although the second gradient need not contain an experimental sample. We will set the centrifugation speed at 30,000, 35,000 or 45,000 rpm for 6 or 12 h. After centrifugation, we will characterize the bottom layer to evaluate the results from different centrifugation settings. HMI will be used to evaluate the centrifugation results and setting parameters through the visualization and characterization of nanoparticles. If too many large blocks are found remaining in the precipitate, higher rotation speed or more centrifugation rounds will be chosen. If we can barely observe particles by HMI, lower parameters will be used. We anticipate obtaining uniformly distributed particles in the final experimental solution. Then, we will optimize centrifugation speed and time based on these characterization results. After the isolation process, we will utilize dialysis to remove the sucrose. Large aggregated blocks will be removed through filtering. The main components we need are the nanoparticles that may contain potential AD biomarkers. Finally, the filtered nanoparticles will be introduced into the f-PNPs arrays.

After binding with the f-PNPs, isolated proteins can be simultaneously analyzed through different detection areas of the f-PNPs array within a short time. Both fluorescence signal and nanomorphology properties will be obtained to generate patient-specific signatures that reflect AD status. In addition, sampling at multiple time points will be conducted to monitor the dynamic changes of the signals. The proposed approach can be performed rapidly with limited amounts of human serum, providing a precision and personalized diagnosis of AD. This proposed research will employ additive nanomanufacturing of f-PNPs and develop the on-chip f-PNPs array for simultaneous multi-biomarker detection. The proposed f-PNPs array can significantly reduce the time and cost compared to conventional diagnostic approaches.

Discussion and conclusions

Through this research, we have proposed ideas of both controlled on-chip peptide self-assembly and sensitive detection of multiple AD biomarkers. Structural parameters of the arrayed f-PNPs, including size and arrangement, will be tuned by changing the composition of the chemical reagents.

The on-chip fabrication techniques can be used to optimize for precise control over the nanostructure and optical properties of the f-PNPs arrays. Through integrating the peptide for personalized AD biomarkers, the arrayed f-PNPs will demonstrate not only fluorescence, but also nanomorphology changes reflecting targeted binding with AD biomarkers, which will offer the most effective and personalized detection of AD. This research has also advanced the discovery of combined biomarkers as patient-specific signatures using patients' blood samples, which is regarded as the future of cost-effective AD diagnosis. The on-chip f-PNPs array will mitigate environmental effects and human errors to provide an effective AD diagnosis.

Additionally, integration of doctor's check-up with a computer-aided customized design for lab-on-a-chip f-PNPs array fabrication for patient-specific AD diagnosis is significant. Conventional diagnostics is faced daunting challenge for AD diagnosis. The marriage of conventional diagnosis with modern molecular diagnosis remains a bottleneck in clinic. Through this research, doctor's check-up and the patient's history will be optimally integrated into a customized design lab-on-a-chip array for patient-specific biomarkers, for which computer plays an essential role from design, to manufacturing and through the clinic. Self-assembly of the f-PNPs and the fabrication of the array are also controlled by the computer and performed through additive nanomanufacturing process. During the patient-specific diagnosis, computer-aided data acquisition can point out multiple data points reflecting disease information for diagnosis.

Finally, utilization of customized lab-on-a-chip additive nanomanufacturing to fabricate fluorescent peptide nanoparticles with targeted binding sites for personalized AD diagnosis. During the additive nanomanufacturing process, various types of amino acid monomers will be controlled to feed into the chip and self-assembled into f-PNPs with customized sequences targeting various AD biomarkers. By exploring the processes where peptide architectures are self-assembled under various environmental conditions, the f-PNPs array can be fabricated in molecular scale by controlling on-chip parameters.

The conceptual work is preliminary with intent to introduce novel techniques to the application. Large-scale manufacturing based on the proposed framework requires extensive validation and optimization.

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