MASTER THESIS

CONSTRUCTION OF A MMP SENSITIVE ARRAY ON GLASS FOR MMP-13 DETECTION IN VITRO

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Abstract

Osteoarthritis (OA) is a type of degenerative joint disease characterized by the degradation of articular cartilage and subchondral bones, and associated inflammation, which finally leads to swelling, stiffness and pain in joints. As one of the most important biomarker of OA, the excessive expression of MMP-13 is responsible for the onset of OA at its early development by the degradation of the structure of ECM in the pathological cartilage. Many popular in vivo and in vitro detection and treatment methods are applied on the foundation of targeting to MMP-13. In our research, we designed a simple and effective strategy of constructed a MMP sensitive array on glass with a MMP-13 cleavable peptide linked with fluorescein (FITC), which was expected to have the potential of detection and measurement of the activity level of active MMP-13 in vitro. Two alternative strategies were developed and a primary MMP sensitive array was finally constructed, and the strategy of developing the MMP sensitive array is possible to be applied for the construction of different sensitive arrays for detecting other biomarkers of OA in the future.

Keywords: Osteoarthritis, MMP-13, MMP sensor, MMP cleavable peptide
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<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>FITC</td>
<td>Fluorescein</td>
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<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<td>μCP</td>
<td>Microcontact printing</td>
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<td>MIMIC</td>
<td>Micromolding in capillaries</td>
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<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<td>Dex-TA</td>
<td>Dextran-Tyramine</td>
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<td>Dex-TA-Mal</td>
<td>Dextran-Tyramine-Maleimide</td>
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<td>Mal-PEG-NHS</td>
<td>Maleimide-Polyethylene glycol-N-Hydroxysuccinimide</td>
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<td>PEG-NHS</td>
<td>Polyethylene glycol-N-Hydroxysuccinimide</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>APTES</td>
<td>(3-Aminopropyl) triethoxysilane</td>
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<tr>
<td>APMA</td>
<td>4-aminophenylmercuric acetate</td>
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1. Introduction

1.1. Background of Osteoarthritis (OA)

Osteoarthritis (OA) is a type of degenerative joint disease which is characterized by structural damages to joints including degradation of articular cartilage and the subchondral bone, and associated inflammation, which finally leads to swelling, stiffness and pain in joints [3-9]. It is estimated that more than 10% of elderly individuals over 60 years suffer from OA worldwide and related healthcare costs over 185 billion dollars per year [1, 2].

In normal situations, there is an equilibrium existing between the synthesis and degradation of the components in extracellular matrix (ECM) secreted by chondrocytes of articular cartilages [3, 4]. However in OA state, the balance between the two opposite process is disrupted abnormally because of inherited disorder or joint injury [5, 6]. The disruption causes a series of severe problems on cartilages including progressive degradation of cartilage tissue such as destruction of ECM components, hypertrophy of chondrocytes, oxidative stress on cellular environment and apoptosis of cells [7, 8]. Apart from the effects on cartilage, the loss in cartilage tissues can also lead to the formation of sclerosis and osteophytes in subchondral bones [9].

There are three main pathological mediators which play important roles in the development of OA. In OA situation, chondrocytes are abnormally activated and produce catabolic factors including pro-inflammatory cytokines (e.g. IL-1, IL-6), catabolic growth factors (e.g. FGF-2, EGF), which are responsible for chondrocyte catabolism [10, 11], and proteases degrading extracellular matrix (e.g. MMPs, matrix metalloproteinases) [12]. The fragments of ECM in the synovial fluid can further induce the catabolism of chondrocytes [13]. During the process of OA, some chondrocytes aberrantly express collagen type X inducing their hypertrophy [14]. Additionally, the loss of cartilage tissues caused by chondrocyte apoptosis and ECM destruction elicits the pathological remodelling in the structure of subchondral bone and forms sclerosis and osteophytes [9, 15]. Since OA is mainly caused by the disruption in equilibrium between catabolic and anabolic processes of ECM, the effective therapeutic method should be able to restore the disrupted balance. In addition, numerous types of factors inducing the progress of OA, such as pro-inflammatory cytokines and ECM degrading proteases, can be applied as biomarkers for the detection and monitoring of OA.
development.

1.2. Functions and dynamics of MMP-13 in the progress of OA

In the main pathological mediators of OA, research have been focused on the family of matrix metalloproteinases (MMP family) in recent years. Among all types of MMPs associated with the development of OA, MMP-13 has a promising potential for the detection and treatment of OA as one of its most important biomarkers. The expression level of MMP-13 is correlated with the degree of hypertrophic differentiation of chondrocytes in pathological situations at the early stage of OA [16]. The overexpression of MMP-13 can lead to the excessive degradation of extracellular matrix of the pathological cartilage and further induce the progress of OA at its early stage [17]. Since the level of MMP-13 is up-regulated significantly at the early stage of OA and can hardly be detected at the late strategy of OA development or in the normal cartilages of adults [18], it has been opted as a powerful biomarker for the diagnosis of OA at its early strategy to increase the rate of preventing further damage of structures in pathological cartilages.

1.3. Applications of MMP sensitive peptides for detecting and monitoring OA development

In recent years, special natural or artificial peptides with particular sequences which are able to be targeted and cleaved by a broad or a particular type of MMPs have been applied as a tool to detect the existence of OA and monitor its developmental level [19-26]. The peptides are often conjugated with dyes (such as fluorophores), and can be targeted by corresponding types of MMPs and reflect their activity level by the changes of the intensity of the dyes when cleaved.

A popular fluorogenic probe for the detection of OA development currently is composed of two different parts, including a fluorescence resonance energy transfer (FRET) pairs and a MMP cleavable peptide as the linker between them [19-24]. The FRET pairs are normally constituted by a donor chromophore and an acceptor chromophore [19]. A fluorescent near-infrared (NIR) dye is always used as the donor chromophore, and its acceptor chromophore is a quencher of its fluorescence. In this probe for imaging the development of OA in vivo, the NIR dye, Cy 5.5, is quenched by its dark quencher called black hole quencher-3 (BHQ3) when they are linked by the
MMP cleavable peptide [20]. The peptide is cleaved when there are certain concentrations of MMPs existing in the environment and the FRET pairs are separated from each other. The near-infrared fluorescence of Cy 5.5 can be detected without the quenching of BHQ 3. Since the intensity of the fluorescence is proportional to the level of expression of MMPs, it can be applied as the reference of reflecting the progress of OA development [21, 22].

The applications of the FRET-based peptide probe were mainly focused on the detection of OA development at early stage in vivo in recent 10 years [20-24]. Since the probe itself cannot exist in circulation and pathological sites of a patient for a long period, it is always conjugated with a relatively stable carrier which is biodegradable and biocompatible, to enhance its stability and accumulation at tissues with OA. Aroa et al. has constructed a MMP-13 sensitive probe conjugated with a biodegradable and biocompatible polymer carrier called poly-l-glutamic acid (PGA), which can exist steadily in vivo and able to monitor OA at its early stage before the structural damage of the cartilage [23]. The imaging ability of the probe can also be applied as a guidance for the therapy of OA by its combination with associated drugs. In recent work, Haimin Chen et al. combined NIR dye labelled MMP-13 sensitive peptides with ferritin nanocages conjugated with cartilage targeting peptides and encapsulating an anti-inflammatory drug. The cartilage targeting peptides can help increasing the accumulation of the nanocages in the cartilage, and the fluorescence of the probes can then demonstrate the pathological sites with OA and its severity [24]. However, the application of the probe in vivo has a limited selectivity against a particular type of MMPs (such as MMP-13) due to the homologous in the structure of catalytic domains of MMP family. In addition, the measurement of the fluorescence intensity released in vivo is needed to be taken by professional optical imaging instruments, and can be only applied to small animals and superficial human tissues due to the current limitation of measurable depth in vivo [20, 23].

To overcome the limited selectivity of the FRET-based peptide probes, the peptide sequences are needed to be improved to increase their specificity to particular types of MMPs. And to overcome the drawback of limited measurable depth in vivo, the probe can also be applied to in vitro detection of the body fluid extracted from OA patients. Ju Hee Ryu et al. has developed a method for the in vitro detection of OA with high selectivity to different types of MMPs [25, 26]. They constructed a diagnostic kit, in which FRET-based peptide probes containing a MMP-13 sensitive peptide were
immobilized into a 96-well plate. The diagnostic kit had a high selectivity to MMP-13, and can detect different activity levels of MMP-13 in the synovial fluid of OA patients at different stages of OA development [25]. By immobilizing peptide linkers with selectivity to different types of MMPs into the probes, their diagnostic kit can be extended to detect 5 different types of MMPs simultaneously [26]. The in vitro application of the FRET-based peptide probes with peptide linkers of different selectivity can partly solve the issues of their in vivo application, however, the process of synthetizing FRET-based peptide probes with required peptide sequences is complicated [25], and commercial FRET-based peptide probes are expensive.

1.4. Application of microcontact printing for constructing in vitro diagnostic tools

Microcontact printing (μCP) is a method to pattern materials with a scale of micrometres and even nanometres on a substrate. Patterns of micro- or nano- scales are constructed on the surface of an elastomeric material, always polydimethylsiloxane (PDMS), to form a stamp before the microcontact printing. A silicon wafer is firstly patterned by photolithography [27], and the PDMS in liquid form is then covered on the patterned surface of the wafer and solidified so that the patterns are transferred to the surface of the PDMS. The material needed to be printed is then adsorbed to the patterned surface of the stamp, and the stamp is finally pressed to a substrate and transfers to be printed material to the surface of the substrate in the same shape and scale as the patterns on the stamp. Since the scale of printed material patterns can be under 50 nm [28], the technique is popular to be used to construct micropatterns of particular biological materials on substrates for diagnostic applications in vitro.

Microcontact printing has been applied to prepare micropatterns of biological materials (such as fluorophore labelled peptides or antibodies) on particular substrates (such as gold and glass), to construct detection tools with high sensitivity. J. P. Renault et al. used microcontact printing to construct micropatterns of a few types of single protein molecules as the foundation of diagnostic detections [29]. In the research of M.E. Hasenbeina et al., peptides available to target osteoblasts were immobilized on glass by microcontact printing for the detection and selection of osteoblasts in their samples [30]. To avoid contaminations of physically adsorbed biomaterials to the prepared
micropatterns by μCP, high molecular weight of biocompatible polymers, such as PEG, are applied to fill the non-printed areas between the micropatterns. The work of Gabor Csucs et al. indicated that the backfilled PEG-grafted copolymers were effective to provide high resistance to the physical adsorption of cells [31]. In recent years, the μCP prepared micropatterns have been combined with microfluidic systems for clinical screening of biomarkers in patients’ body fluid. Shivani Sathish et al. combined a microfluidic system with micropatterns of antibodies of human interleukin 6 and human c-reactive protein in their work, and successfully detected them in fluidic samples [32].

1.5. Project outline

The aim of my research was to create an effective tool for the detection of the activity level of a certain type of MMP in vitro. In detail, we have investigated efficient methods to construct a MMP sensitive array for detecting the activity level of MMP-13 in vitro. The MMP sensitive array was constituted with a type of MMP-13 cleavable peptide labelled with fluorescein (FITC) on its tail (Figure 1 A) [33, 34], and the substrate of the array was glass. As mentioned above, since the overexpression of MMP-13 happens at the early stage of OA development and its expression level decreased with the progress, the MMP sensitive array was designed to have the potential of measuring the activity level of MMP-13 in the body fluid of patients so the signal of OA development can be detected at its early stage. We did not choose a FRET-based peptide probe because commercial FRET-based peptide probes take high costs. In addition, the principle of the detection of our MMP sensitive peptide works opposite to the popular FRET-based peptide probe: the activity level of the tested MMP-13 is indicated by the decrease of the fluorescence intensity when the peptides in the array are cleaved. This is a novel idea that we have developed in this research.

Two different strategies of constructing a MMP sensitive array on glass were explored in this research. In the first strategy, the peptide-FITC was modified directly onto the surface of glass. Two different modification methods of the peptides were tested, including droplets of certain concentrations of peptides and microcontact printing of the peptide with PDMS stamps on the surface of the glass modified with maleimide groups. The maleimide can form a covalent bond with the C-terminus cysteine of the peptide (Figure 1 B), and to decrease physically adsorbed peptides,
the maleimide was modified onto the glass surface via a PEG-NHS ester. We chose the microcontact printing method because the size and fluorescence intensity of each micropattern was uniform and easily measured. Glass slides were chosen as substrates due to their low costs and the fluorescence of an array immobilized on it can be easily measured under fluorescent microscopes.

Since we did not achieve a MMP sensitive array due to the low intensity of fluorescence of modified peptides, we adopted another strategy to enhance the intensity. In the second strategy, a layer of hydrogel, which was formed by the crosslinking of dextran-tyramine polymers with maleimide moieties, was firstly modified on the surface of glass, and then the peptides were reacted with the maleimide moieties of the hydrogel to create the points of the array. The structure of dextran-tyramine polymers and the process of their crosslinking are shown in Figure 1 C. Dextran is the backbone of the polymer, and the tyramine groups modified on the dextran are used as the crosslinker of the polymers. In the presence of hydrogen peroxide (H$_2$O$_2$) and the enzyme horseradish peroxidase (HRP), the polymers can form stable and biocompatible hydrogels which can be directly applied to provide mechanical supplementation to the OA suffered cartilage structure and so facilitate further repair to the damaged structure [36, 37].

Since the maleimide groups were immobilized to the backbone of dextran-tyramine polymers, and the dextran-tyramine polymers can form stable connections with the modified glass surface, all the maleimide groups on polymers were fixed to the glass surface when the hydrogel formed. Compared with the monolayer of maleimide modification in the first strategy, the amount of maleimide groups was drastically increased and they existed throughout the gel layer, which were able to catch more peptides and enhance the total fluorescence intensity.

The hydrogels were modified in the form of a whole layer or strips of microscales on the glass surface, and the peptide solution was then covered upon the hydrogel or spotted into the hydrogel. Available MMP sensitive arrays were constructed in the second strategy and applied for testing the activity level of MMP-13. The prepared MMP sensitive array was finally incubated with different concentrations of MMP-13, and the decrease of the fluorescent intensity of the array during a fixed period can reflect the activity level of a certain concentration of MMP-13.

Compared with the popular techniques for in vitro detection of OA, our methods of constructing the
MMP sensitive array is very simple and cost-effective. Meanwhile, the application of dextran-tyramine based hydrogels for enhancing the immobilization of peptides on glass, and the detection of MMP-13 activity by decreased fluorescence intensity of the peptides are novel strategies for designing MMP detectable tools in vitro.

A. 

\[
\text{FITC-AhxGRDGPQG1WQDRC-OH}
\]

B. 

![Diagram of peptide and maleimide reaction]

C. 

![Diagram of dextran-tyramine and crosslinking process]

**Figure 1.** A. The sequence of the MMP sensitive peptide with a dye of FITC we used. The red arrow shows the cleavage site of MMP-13 on the peptide. B. The reaction between the peptide and maleimide modified substrates. The thiol group in the Cysteine of the peptide can react with maleimide under pH 6.5 to 7.5 and attach the peptide on the glass or hydrogel. C. The structure of dextran-tyramine and process of crosslinking of dextran-tyramine. The reaction was finished under the existence of hydrogen peroxide (H₂O₂) and the enzyme horseradish peroxidase (HRP) [35].
2. Materials and methods

2.1. Materials

Glass slides (length: 75 mm, width: 26 mm, Thermo Fisher Scientific); Coverslips (22.1 mm, Thermo Fisher Scientific); H₂SO₄ (98%, Sigma-Aldrich); H₂O₂ (30%, Merck KGaA); (3-Aminopropyl)triethoxysilane (APTES) (99%, Sigma-Aldrich); Maleimide-Polyethylene glycol-N-Hydroxysuccinimide Ester (Mal-PEG-NHS ester) (2 KDa, Creative PEGWorks); Polyethylene glycol-N-Hydroxysuccinimide Ester (PEG-NHS ester) (5 KDa, Creative PEGWorks); NaHCO₃; 3-(4-Hydroxyphenyl) propionic acid N-hydroxysuccinimide ester (263.25 Da, Sigma-Aldrich); Dextran-Tyramine (40 KDa, 10% tyramine, prepared in our lab); Dextran-Tyramine-Maleimide (58.88 KDa, 8.5% tyramine and 3.25% maleimide, prepared in our lab); HRP (250 U/ml); L-cysteine (121.16 Da, Sigma-Aldrich); SYLGARD™ 184 silicone elastomer Base (Dow Europe GmbH); SYLGARD™ 184 silicone elastomer Curing agent (Dow Europe GmbH); Peptide-FITC (2047.2 Da, Pepscan Presto); Recombinant human MMP-13 (52 KDa, R & D Systems); 4-aminophenylmercuric acetate (APMA) (≧ 90%, Sigma-Aldrich); Trisma-base (121.14 Da, Sigma-Aldrich); CaCl₂; NaCl; Brij 35 (30% w/w in water, Merck KGaA).

2.2. PDMS stamp preparation

A cleaned silicon wafer with two different micropatterns or one layer of tape (approximately 1.5 mm in width and 40 μm in thickness) on its surface, was prepared as a mold by surrounding a tape around it. The mixture of PDMS base and its curing agent (the ratio between base and curing agent was 10:1 w/w) was poured onto the mold and maintained to cure at 70 °C overnight [38]. The cured PDMS was finally removed from the mold and cut to stamps with appropriate sizes.

2.3. APTES modification on glass surface

The glass slides or coverslips, as substrates of our MMP sensitive array, were firstly immersed into Piranha solution (98% H₂SO₄ and 30% H₂O₂ in a ratio of 3:1 v/v) for 1 hour at room temperature. Subsequently, the glasses were washed with Milli-Q water twice and ethanol twice successively, then dried with nitrogen. APTES was dissolved sufficiently in toluene (5% v/v) and the glass was immersed in the mixture for 1 hour (the glasses should be avoided overlapping on each other).
Finally, the APTES modified glasses were immersed in toluene and shaken for 15 minutes, followed by sonication in ethanol for 10 minutes and dried under a nitrogen flow [39].

2.4. Modification of conjugated maleimide-PEG on glass surface

In the first strategy of the research, the next step was the modification of maleimide groups on the glass surface. To react Maleimide-PEG-NHS ester with the amine groups (involved in APTES) on glass surface, the reaction buffer was firstly prepared (0.1 M of fresh sodium bicarbonate buffer, pH 8.5. 84 mg NaHCO₃ was dissolved in 10 ml of Milli-Q water and the pH was not needed to be adjusted). Maleimide-PEG-NHS ester was dissolved in the reaction buffer to prepare certain concentrations of reaction solution (3 different concentrations of maleimide-PEG-NHS were used: 1 mM, 10 mM and 30 mM). The reaction solution was covered on the surface of the glass immediately and reacted for 1 hour or 3 hours. Thereafter, the glass surface was rinsed with copious amounts of Milli-Q water. PEG-NHS ester, as the control, was also modified on a glass surface with the same method [40].

2.5. Water contact angle test of glass surface in different strategies

For non-treated glass slides, glass treated with piranha solution, glass modified with APTES, and glass modified with maleimide-PEG-NHS (or PEG-NHS), the water contact angles of their surfaces were tested respectively. For each test, the water contact angles on one glass slide were measured in triplo and their mean value was taken as the result. This gave an indication whether the functionalizations were successful.

2.6. Modification of hydrogel layers with maleimide moieties on glass surface

In the second strategy, to immobilize dextran-tyramine-maleimide on a glass surface, 3-(4-Hydroxyphenyl) propionic acid N-hydroxysuccinimide ester was firstly reacted with APTES modified glass. It was dissolved in DMSO and then mixed with 0.1 M sodium bicarbonate buffer (final concentration was 1 mg/ml) [40, 41] before covered on a glass surface.

Dextran-tyramine-maleimide polymers were dissolved in PBS (50 mg/ml) containing HRP (1.6 % v/v), and subsequently mixed with H₂O₂ (final concentration of H₂O₂ is 0.0025 %) [35] on ice. Two
alternative methods were applied for the immobilization of the hydrogel layers on a ice-cooled glass surface. The first method was called micromolding in capillaries (MIMIC) [42], in which a PDMS stamp with capillaries was placed on a glass surface, and the dextran-tyramine-maleimide solution prepared on ice was absorbed in the slots of the capillaries under capillary action. In the second method, a PDMS stamp (no pattern) was pressed on a droplet of dextran-tyramine-maleimide solution on a glass surface. The polymers formed hydrogels and were crosslinked with the modified phenol groups on glass surface at room temperature. The reaction proceeded for 20 minutes and the glasses were immersed in Milli-Q water for 10 minutes to remove PDMS stamps and then shaken in Milli-Q water for 30 minutes to remove residual H₂O₂. Prior to peptide conjugation, the hydrogels can be stored in Milli-Q water at 4 ℃ for no more than 2 days.

2.7. Peptide-FITC modification on maleimide-PEG modified glass surface

In the first strategy, MMP sensitive peptide-FITCs were immobilized directly onto the maleimide-PEG functionalized glass surface. The prepared peptide-FITC solution (in PBS) was dropped directly onto the modified glass surface (0.5 μl/droplet). The glass was kept in high humidity and maintained at 4 ℃ overnight. Afterwards, the residual droplets were removed by a clean paper tissue and unreacted maleimide groups were blocked with 50 mM L-cysteine (in PBS) for 30 minutes. The glass was finally rinsed with copious amounts of Milli-Q water.

Alternatively, microcontact printing was applied. Before doing the microcontact printing, the PDMS stamps were treated with oxygen plasma. A small droplet of peptide solution was placed on the patterned surface of a stamp and maintained for 5 minutes. Afterwards, most solution was removed with a clean paper tissue after the reaction was finished, and the remaining solution was dried with a stream of nitrogen. The stamps were finally placed on the maleimide-PEG modified glass and the patterned surface with the ink of peptides was in contact with glass surface tightly by applying pressure on the stamps. After 30 minutes, the stamps were separated from the glass surface [43], and remaining maleimide groups were blocked with 50 mM L-cysteine for 30 minutes and rinsed with copious amounts of Milli-Q water.
2.8. Peptide-FITC modification on hydrogel modified glass surface

In the second strategy of the research, three methods were applied to modify peptide-FITCs in the hydrogel with maleimide moieties on glass surface. Before the modification with peptides, the surface of the hydrogel was dried with a slight stream of nitrogen while the inside of the hydrogel was kept wet. The first method of dropping peptide solution directly on the hydrogel was similar with the method of direct droplet in the first strategy. The droplets were placed on the surface of the hydrogel for overnight at 4 °C, and the hydrogel was directly blocked with 50 mM L-cysteine after the reaction was finished, followed by shaking in Milli-Q water for 30 minutes to remove residual L-cysteine. The method was subsequently replaced by spotting points of peptides in the hydrogel with a spotter machine in our lab (CFM 2.5 Continuous Flow Microfluidics, Wasatch Microfluidics). The prepared hydrogel on glass was fixed into the spotter, and a certain concentration of peptide solution (20, 10, 5, 2.5 μg/ml) was printed on the hydrogel for 60 minutes. After the printing, the hydrogel was washed with Milli-Q water for 20 minutes automatically in the machine.

For the MIMIC-prepared hydrogels, the peptide solution (diluted by 5 folds from original solution, so modified concentration was 200 μg/ml) was covered on the strips of hydrogels on glass surface, reacting overnight at 4 °C and followed by shaking in Milli-Q water for 30 minutes to remove unreacted peptides in the residual solution (blocking was not needed).

2.9. Measurement of the quality of MMP sensitive array

The quality of MMP sensitive array constituted by peptide-FITCs after their modification was tested by using a fluorescent microscope (EVOS FL Color Imaging System, Thermo Fisher Scientific). The size and shape of the patterns of peptides, and most importantly, the fluorescence intensity of the array were measured with image J from the picture taken by the microscope and the data was collected and treated with Origin 2018.

2.10. Activity level test of MMP-13

The MMP sensitive array on the glass was placed in the well of a 12-well plate. Before the incubation with MMP-13, the glass was washed sufficiently for one day at 37 °C to remove all peptides which were not attached to the glass surface. We used MMP assay buffer, which was applied for supplying a reactive environment for MMP-13, to wash the glass. The buffer contained
50mM Trisma-base, 10mM CaCl₂, 150mM NaCl, and 0.05% (w/v) Brij 35 (adjusted to pH 7.5). MMP-13 was activated before reacting with the MMP sensitive array. Original solution of MMP-13 was diluted in MMP assay buffer to 50μg/ml and APMA was added until the final concentration of MMP-13 was 1 nM. The mixture was incubated at 37 °C for 2 hours to activate MMP-13 [44]. 2 ml of activated MMP-13 was incubated with MMP sensitive array at 37 °C in a temperature-controllable shaker. The variation of the fluorescence intensity of MMP sensitive array was measured per hour.
3. Results and Discussion

3.1. Direct modification of Maleimide-PEG on a glass surface

In the first strategy of the research, maleimide groups applied for the immobilization of prptide-FITC were modified directly on the glass surface. APTES with amine groups (-NH₂) was firstly reacted with the surface of a glass which had been treated with piranha solution. Maleimide-PEG-NHS esters (or PEG-NHS esters) in different concentrations were then reacted with the APTES modified glass for different periods. The process of direct modification is present in Figure 2 A. To check the level of different reactive groups modified on the glass surface at different strategies (hydroxyl in piranha solution modification, amine groups and maleimide or PEG groups), water contact angel tests were applied in different phases of glass modification. The results are shown in Figure 2 B.

In the results of water contact angle test, water contact angle on glass surface decreased after modification with piranha solution and increased drastically after APTES modification, indicating that hyroxyl (hydrophilic groups) and amine groups (hydrophobic groups) were successfully immobilized on the glass surface. After the modification of 10 mM of maleimide-PEG for 1 and 3 hours or 10 mM of PEG for 3 different durations, the water contact angel decreased on the glass surface, which means the two types of groups were modified on glass surface because they are hydrophilic. However no matter for maleimide-PEG (1 hour and 3 hours) or PEG (3 periods) modification, the decline of water contact angle were similar and not obvious. Further chemical analysis would be needed to test the surface density of modified maleimide-PEG-NHS or PEG-NHS, to check whether enough maleimide-PEG or PEG was immobilized on the glass surface. For maleimide-PEG modification overnight, the water contact angle increased abnormally compared with APTES modified glass surface by unclear reasons.
A.

1. Glass modification in the first strategy. Step ① is the APTES modification, in which amine groups react with hydroxyl modified by piranha solution on glass. Step ② shows the process of direct modification of maleimide-PEG to a glass surface. The -NHS group in a maleimide-PEG-NHS ester or PEG-NHS ester reacts with amine groups of APTES.

B. The results of water contact angle test for different strategies of modification on the glass surface. The tested concentration of maleimide-PEG-NHS or PEG-NHS is 10 mM.

3.2. PDMS stamp preparation

Before the microcontact printing and MIMIC, the PDMS stamps with micropatterns were firstly prepared. PDMS mixture was poured onto a cleaned silicon wafer-based mold and cured, and then cut to appropriate pieces of stamps. The process of preparing PDMS stamps, and pictures of silicon wafer-based mold and patterned PDMS stamps are shown in Figure 3.
Figure 3. A. The structures of the patterned surfaces of silicon wafers, and the process of making PDMS stamps. Red dotted boxes show the location for the knife cutting. B. Pictures of a silicon wafer and the mold, and two different types of PDMS stamps prepared. The diagrams of the shapes of micropatterns on the surface of the wafer or PDMS stamps are shown beside.

3.3. Immobilization of peptide-FITC on a maleimide-PEG modified glass surface

Two different methods including direct droplet and microcontact printing were applied to immobilize peptide-FITCs on glass surface modified with maleimide-PEG-NHS. The processes of the two different methods are shown in Figure 4. In the method of direct droplet, the prepared peptide solution was dropped directly to the modified glass surface with a pipette. In the method of microcontact printing, the peptide solution was firstly inked on the surface of prepared PDMS stamps with small patterns of micrometre scale. The stamps were then placed on the modified glass surface and the peptides inked on stamps can be fixed to the glass surface in a short period. The results of peptide modification were checked under fluorescent microscopy, and the fluorescence
intensity of results was measured with image J. The pictures of the results of two different modification methods, and corresponding data of their fluorescence intensity are shown in Figure 5.

A. 

![Diagram A](image)

B. 

![Diagram B](image)

**Figure 4.** A. Direct droplet modification of peptide-FITC on the glass surface. B. Microcontact printing of peptide-FITC on glass surface with PDMS stamps. For both methods, the same concentration of peptide solution was modified on three different modified glasses with maleimide-PEG-NHS, PEG-NHS or just APTES and reacted for the same period.

In the results of direct droplet modification, for the three different concentrations of maleimide-PEG treated groups, the fluorescence intensity on 30 mM maleimide-modified glass surface was the highest, indicating the amount of maleimide immobilized in this group was the most. Among the three PEG treated groups, the fluorescence intensity of 30 mM PEG-modified group was lowest and had no differences with the 10 mM PEG-treated group. Since PEG has the ability to avoid physical adsorption of the peptides on the glass surface [40, 45], it is indicated that most amount of PEG was modified in 30 mM group, and the peptides physically adsorbed to glass surface was the lowest. So the physical adsorption of peptides in 30 mM maleimide-modified group was also the lowest among three maleimide-treated groups, and the group had the most amount of chemically attached peptides.

Compared with the control group on which only physically adsorbed peptides existed, the fluorescence intensity of 30 mM maleimide-modified group was higher and 30 mM PEG-modified group was lower as expected, but the increase or the decline was not dramatic. The possible reason is only a really small amount of maleimide-PEG-NHS or PEG-NHS has been immobilized to glass surface due to the high molecular weight of the two esters.
Figure 5. A. The results of direct droplet modification. 3 different concentrations of maleimide-PEG or PEG had been immobilized on glass surface for 1 hour before the peptide modification. Modification of peptides were same for all glass slides. B. The results of microcontact printing. Two different patterns were applied and their results are shown in ① and ② respectively. The concentration of maleimide or PEG was 10 mM, treated on glass surface for 1 hour or 3 hours. Modified concentration and reaction time of peptide were same for all areas. Scale bar: 400 μm.
In the results of microcontact printing, for the pattern of points, there was an abnormal decrease in the fluorescence intensity of the group in which maleimide-PEG was reacted for 3 hours, compared with the maleimide-treated group of 1 hour. The fluorescence intensity of the PEG group treated for 3 hours was lower than the PEG group treated for 1 hour, and the fluorescence intensity of both PEG groups was lower than maleimide groups as expected. For the second type of pattern, the fluorescence intensity of the maleimide-treated group for 3 hours was higher than the maleimide-treated group for 1 hour. There were no differences between the fluorescence intensity of two PEG groups, which was also lower than the fluorescence intensity of maleimide treated groups. The trend in the comparisons of fluorescence intensity for the pattern of lines was better than the first type of pattern, although the entire fluorescence intensity of the pattern of lines was lower than the first one.

For both methods of peptide modification, the fluorescence intensity of maleimide-treated groups had no dramatic increase compared with the PEG-modified groups or the control group, and was difficult to be observed under the fluorescent microscope. It means that the MMP sensitive arrays prepared in the methods of the first strategy had no possibility for the next test of MMP-13 activity level. As a consequence, we prepared another strategy to modify much higher amount of the peptides on the glass surface.

3.4. Modification of hydrogel with maleimide moieties on a glass surface

In the second strategy, the first step was still the APTES modification on glass surface. To increase the amount of immobilized maleimide groups, we applied a layer of hydrogel on glass surface, which is formed by a type of polymers called dextran-tyramine-maleimide. An agent called 3-(4-Hydroxyphenyl) propionic acid N-hydroxysuccinimide ester was firstly reacted with modified APTES, which can form crosslinks with tyramine groups on the polymers. Two different methods were applied for the immobilization of the hydrogel layer on glass surface. The first method was called MIMIC and can finally formed strips of hydrogels on glass under capillary action with a micropatterned PDMS stamp. In the second method, a whole layer of hydrogel was modified on the glass surface by the pressure of a PDMS stamp with a flat surface. The processes of the two modification methods, and the pictures of immobilized hydrogel layers are shown in Figure 6.
Figure 6. **A.** The process of glass modification in the second strategy. In step ①, after APTES modification, the 3-(4-Hydroxyphenyl) propionic acid N-hydroxysuccinimide ester was reacted with APTES to prepare sites for immobilization of dextran-tyramine-maleimide constituted hydrogels. In step ②, the mixture of Dex-TA-Mal (or Dex-TA as control), HRP and H₂O₂ was added to the modified glass surface in two different methods on ice. The ice was then removed and hydrogels can form in few seconds. **B.** Pictures of hyrogels on glass in two methods and the microstructure of a hydrogel. Scale bar: 1000 μm.

### 3.5. Immobilization of peptide-FITC in the hydrogel layer

For the strips of hydrogel layers modified by MIMIC, the next immobilization of peptide-FITC in these hydrogels was simple. The prepared peptide solution was just coverd on the hydrogel layers
and maintained until the reaction was finished. For the entire hydrogel layer on glass surface modified with another method, there were two different methods of peptide modification applied.

A.

①

![Diagram 1](image1)

Overnight at 4 C

②

![Diagram 2](image2)

Print: 1h
Wash: 20 min in water

B.

![Diagram 3](image3)

Overnight at 4 C

Figure 7. A. ① is the direct droplet of peptide solution with a pipette on hydrogel. The peptides in droplets can be adsorbed and attached to the maleimide groups overnight at 4 C. ② is the method of spotting points of peptide on hydrogel with the spotter machine. The hydrogel was immobilized to the surface of a coverslip and then the coverslip was fixed in the spotter. Prepared peptide solution was printed on the hydrogel in uniform size and shape. B. On MIMIC modified hydrogels, the peptide solution was directly placed, and peptides were fixed in the area of hydrogel patterns overnight at 4 C.

In the first method, the prepared peptide solution was dropped on the surface of the hydrogel directly with a pipette. Since a high concentration of peptide was needed and the size of modified peptide points on hydrogel can not be controlled, a spotter machine in our lab was applied to print points of peptide on the hydrogel instead of using a pipette. In this method, only a small concentration of peptide solution (1/5 of the old method) was needed, and the size and shape of all peptide points printed was uniform. The processes of the three modification methods are shown in Figure 7. The results of peptide modification in hydrogel were checked by fluorescent microscopy and measured with image J, which are shown in Figure 8 and 9.
Figure 8. A. Results of direct droplet modification of peptides on hydrogel with a pipette. Peptide solution was modified on three different hydrogels constituted with: dextran-tyramine-maleimide (Dex-TA-Mal), dextran-tyrymine-maleimide blocked by 50 mM L-cysteine and dextran-tyramine (no maleimide). Scale bar: 1000 μm. B. Results of printing of peptide solution on hydrogel with the spotter machine. Four different concentrations of peptide solutions were tested on three different hydrogels. Scale bar: 400 μm.

In the results of the direct droplet modification with a pipette, the fluorescence intensity of the immobilized peptide points in hydrogel constituted with dextran-tyramine-maleimide was
drastically higher than the fluorescence intensity of the blocked Dex-TA-Mal group and Dex-TA group. The fluorescence intensity in the Dex-TA-Mal group can also be observed clearly under the microscope, and almost no fluorescence intensity can be observed in another two groups. Since the blocked Dex-TA-Mal group and Dex-TA group can reflect the fluorescence intensity of peptides physically adsorbed by the Dex-TA-Mal itself or the Dex-TA backbone respectively, it is indicated that a majority of peptides were chemically attached to the maleimide groups in the hydrogel of Dex-TA-Mal, and the physically adsorbed peptides can be ignored. The results is acceptable for the next activity level test of MMP compared with the results of the first strategy, but the problems was that it was impossible to control the size and shape of the peptide points.

In the results of the printing modification with the spotter machine, four different concentrations of peptide solution was immobilized in the three groups of hydrogels to find out the best concentration for the modification. The fluorescence intensity of peptide points increased with the rise of the peptide concentration. For the peptide concentrations of 20 and 10 μg/ml, the peptide point in the Dex-TA-Mal group had an obviously higher fluorescence intensity than the blocked Dex-TA-Mal group and Dex-TA group. Compared with the modification with a pipette, the fluorescence intensity of 20 and 10 μg/ml peptide solution modifications can also be observed clearly but the applied concentration of peptide solution was decreased by 10 folds and 20 folds respectively. In addition, the size and shape of all spotted points were uniform and each point was approximately 1/8 of the points in the last methods in size. The final concentration of peptide solution we decided to be applied for constructing the MMP sensitive array was 20 μg/ml.

![Figure 9](image)

**Figure 9.** Results of peptide modification on MIMIC prepared hydrogels. Scale bar: 1000 μm.
In the results of the MIMIC modification, although the fluorescence intensity of the Dex-TA-Mal group was the highest among all three groups, the difference of the fluorescence intensity between Dex-TA-Mal and Dex-TA modified glass slides was not dramatic, and the entire fluorescence intensity of both groups was really low. The possible reason was that the thickness of the hydrogels was too low so that the total amount of maleimide groups on unit area of each strip was really small. The fluorescence intensity of the blocked Dex-TA-Mal group can not be observed because the immobilized L-cysteine in hydrogel can block the adsorption of the peptides.

Compared with another two methods, the properties of peptide points prepared by the spotter machine was ideal, so the MMP sensitive array was finally constructed with this method for the next activity level test of MMP-13.

### 3.6. Optimization of the quality of MMP sensitive array

One problem existing in the prepared MMP sensitive array was that the quality of constructed MMP sensitive array was difficult to be controlled, meaning that the results of MMP sensitive array with a high quality was difficult to be reproduced. To solve this problem, we have prepared several PDMS stamps with a slot of the same thickness on one surface. The PDMS stamps were then applied to prepare hydrogel layers with the same thickness on the glass surface, and the process was like the MIMIC method, which is shown in Figure 10 A. The pictures of the PDMS stamps, the results of prepared hydrogel layer and printed peptide points on it are also shown in Figure 10 B. Unfortunately, the fluorescence intensity of the printed peptide points were quite weak. The possible reason was still needed to be investigated.
3.7. Activity level test of MMP-13 with prepared MMP sensitive array

A certain concentration of activated MMP-13 was incubated with the MMP sensitive array prepared by the spotter machine, and the decrease of the fluorescence intensity of the peptide points in the array can reflect the activity level of tested MMP-13. The processes are shown in Figure 11.

Figure 10. A. The schematic of the preparation of a hydrogel layer with new PDMS stamps. The picture shows the slot on the surface of the stamp. Scale bar: 400 μm. B. The picture of the hydrogel layer before printing with the spotter machine and the spotted peptide points on the hydrogel. The concentration and reaction time were the same with experiments before. Scale bar: 1000 μm.

Figure 11. A. The washing step of MMP sensitive array modified glass. The glass slides were loaded in the wells of a 12-well plate and MMP assay buffer, which can supply a reactive environment for MMP-13, was used as washing solution. B. Activity level test of MMP-13. Activated MMP-13 was incubated with MMP sensitive array to test its activity level. In another group, the array was incubated with MMP assay buffer as control.
Before the incubation with MMP-13, the prepared MMP sensitive array on glass was washed sufficiently to remove most physically adsorbed peptides remaining in the hydrogel layer. The cleaned MMP sensitive array was then incubated with activated MMP-13 and reacted on a shaker at 37 °C. The change of the fluorescence intensity was recorded per hour under fluorescent microscopy and measured with image J. The results are shown in Figure 12.

There was no dramatic decrease in the fluorescence intensity of the MMP sensitive array in the MMP treatment group during the 5 hours of incubation with MMP-13. There are several possible reasons leading to this situation and will be discussed in the following contents.

**Figure 12.** The results of activity level test of MMP-13. The total period of the test was 5 hours, and the pictures of the reaction for 1 hour, 3 hours and 5 hours are shown. Scale bar: 1000 μm. In the chart, the fluorescence intensities at different reaction time were normalized as the percentages accounting for the original fluorescence intensity of the tested MMP sensitive array.

### 3.8. Discussion and recommendations

For the first strategy in my research, the main problem was that the fluorescence intensity of the peptide points or patterns was hardly to be observed under fluorescent microscopy. The main reason causing this situation, as analyzed in the results, was that the bulkiness of maleimide-PEG-NHS or PEG-NHS molecules blocked their reaction with amine groups of APTES due to their large molecular weight. One method to solve this problem is using a lower molecular weight of maleimide-PEG-NHS ester or another small molecular weight of maleimide-conjugated ester.
Maleimide-PEG-NHS ester has a linear structure with a maleimide group and a -NHS group at two ends respectively. Since the PEG part contributes to the large molecular weight of the Maleimide-PEG-NHS ester, the -NHS groups have few chance to get in touch with the amine groups modified on a glass surface. The work of Jacopo Movilli et al. has supplied a novel method. They used a polymer called poly-L-lysine (PLL) which has a linear structure and branches with an amine group at the end. By reacting with a small molecular weight of maleimide-linked ester, a certain percentage of branches in a polymer can be modified with maleimide groups. Compared with our applied maleimide-PEG-NHS ester, they applied a maleimide-conjugated ester with a much smaller molecular weight, and the PLL backbone is effective to avoid the physical adsorption of DNA or proteins [42, 46]. The PLL polymer as well as the small molecular weight of maleimide-conjugated ester are hopeful to be applied for improving the fluorescence intensity on the modified glass by replacing the direct modification of maleimide-PEG-NHS esters.

No matter using the maleimide-PEG-NHS or the small molecular weight of maleimide-linked ester, there is only a monolayer of peptide modified on the surface of the substrate finally. This is also a possible factor to limit the total fluorescence intensity. Another method to increase the fluorescence intensity of the array is increasing the modified layer of peptide-FITCs. In the work of Mario Beyer et al., the polymerization of a monomer called PEG methacrylate (PEGMA) was induced on modified surface of glass slides. The polymer formed by PEGMA has a brush-like structure with several layers of branches for targeting cysteines in a peptide. Since there were several layers of target sites for the peptides, the total amount of modified peptides was available to be increased and their fluorescence can be easily observed [47]. The idea of modifying several layers of peptides on a glass surface was similar with the idea of our second strategy. The method can be applied in the construction of our MMP sensitive array due to the available attachment of our peptide-FITC to the target sites of the polymer.

For the second strategy of my research, the primary MMP sensitive array on the glass substrate has been successfully constructed. The most important issue needed to be solved is that the prepared MMP sensitive array can not detect the activity of incubated MMP-13. There were several factors causing this problem. The first possible reason was that the activity of the MMP-13 can only maintain for a short period under the experimental condition, so only a really small amount of
peptides were cleaved from the array in this duration, and the decrease in fluorescence intensity caused by their cleavage can be ignored. The second possible reason was that the crosslinked structure inside the hydrogel limited the movement of MMP-13 and the release of cleaved peptide fragments. It is more difficult for MMP-13 to target the peptides on array compared with in a liquid environment, and the cleaved peptides can not be released from the hydrogel sufficiently during the test period. The final possible factor was that the concentration of MMP-13 applied in the test was relatively low for the array, so that the sensitivity of our array was not enough to detect their activity. In the future work, the existing period for MMP-13 in current experimental situation is needed to be tested, and the incubation time should be prolonged for sufficient reaction between MMP-13 and peptides and release of cleaved peptide fragments. The baseline of the sensitivity of the array should also be tested to demonstrate the lowest detectable concentration of MMP-13.

Another problem existing in the results of the second strategy was that the non-uniform quality of constructed MMP sensitive array as mentioned before. The main reason for this problem, we considered, was the inhomogeneous thickness of the hydrogels of different samples. Since the size of peptide points printed by the spotter machine was uniform, the only factor influencing the final modified amount of peptide was the thickness of hydrogels. At the end of our improvement, the problem needed to be solved was that the fluorescence intensity was not enough to be observed. We planned to improve the fluorescence intensity by increasing the thickness of the hydrogel layer in the future.
4. Conclusion

4.1. Direct modification of MMP sensitive array on a glass surface

My research was separated into two different strategies. In the first part, the goal was to construct the MMP sensitive array on glass directly with a MMP-13 sensitive peptide. To attach the MMP-13 sensitive peptide-FITC on glass surface, we firstly immobilized APTES with amine groups on the glass surface to obtain amino functionalized glass. Subsequently, a maleimide-PEG-NHS with a maleimide group as the target site of the peptide-FITC, was reacted with the amine groups on glass surface. Water contact angle test indicated that the amine groups and maleimide groups were immobilized to the surface of the glass.

After the maleimide groups were immobilized to the surface of the glass slide, two different methods were applied to attach peptide-FITC onto the modified glass surface directly. In the first method, the solution of peptide was dropped directly to the modified glass surface. In the second method, PDMS stamps with micropatterns on surface were applied to make microcontact printing for the peptide solution onto the modified glass surface. Unfortunately, we did not achieve an available MMP sensitive array for both modifications because the fluorescence intensity of peptide points modified on glass was not enough to be distinguished by the fluorescent microscope. A possible reason could be the low surface density of the maleimide-PEG-NHS ester modified onto the glass surface due to its linear structure and high molecular weight.

4.2. Modification of the MMP sensitive array on a glass surface, based on the foundation of a hydrogel layer

In the second part of my research, to increase the amount of maleimide groups modified on the glass surface, we modified a hydrogel layer constituted by dextran-tyramine-maleimide polymers before the modification of the peptide. The polymers were crosslinked with each other and the glass surface modified with tyramine groups to for a hydrogel layer on the glass, which consisted several layers of maleimide groups inside. Three different modification methods were then applied to immobilize the peptide-FITC inside the hydrogel layer. In the first method, the solution of the peptide was dropped directly to the surface of the hydrogel layer and maintained until the peptide
solution permeated into the gel layer and attached to the maleimide groups. To control the size and shape of peptide points immobilized to the hydrogel layer, a spotter machine was applied to print uniform peptide points on the prepared hydrogel layer. In the last method of modification, strips of hydrogel layers were firstly modified on glass surface by MIMIC with PDMS stamps and the solution of the peptide was then covered on the strips and maintained until the reaction was finished. Dramatic fluorescence was observed on the MMP sensitive array prepared by the direct droplet and printing with the spotter machine, but the fluorescence intensity of arrays prepared by biomimicking was difficult to be observed. Since the MMP sensitive array prepared with the spotter machine had enough fluorescence intensity and uniform size and shape, it was applied to test the activity level of MMP-13.

4.3. Activity level test of MMP-13 with prepared MMP sensitive array

The MMP sensitive array prepared with the spotter machine was applied to test the activity level of a certain concentration of MMP-13. The MMP sensitive array was firstly washed with MMP assay buffer and then incubated with activated MMP-13 solution. However, no dramatic decrease of the fluorescence intensity on the MMP sensitive array was observed. There were several possible reasons causing this problem, which was associated with the activity of the tested MMP-13, and the structure and sensitivity of prepared MMP sensitive array. In addition, to make the quality of prepared MMP sensitive array controllable, the thickness of the hydrogel layer was normalized with PDMS stamps having same thickness of slots on their surface. The fluorescence intensity of peptide points printed on these hydrogels was unavalaible to be observed, and the possible reason was the thickness of the hydrogel was too small, which will be improved to find a suitable thickness in the future work.

In summary, we designed a simple and effective strategy to construct a primary MMP-13 sensitive array for the in vitro detection of OA development. The MMP sensitive array was expected to be applied for detecting and measuring the activity level of active MMP-13. Although there are still a lot of improvements needed to be investigated for the quality and sensitivity of our MMP sensitive arrays, the strategy of developing the MMP sensitive array has a promising potential be applied for the construction of different sensitive arrays of other biomarkers of OA. For example, aggrecanase
is also a type of extracellular proteolytic enzyme and can induce OA by degrading aggrecans in the cartilage. Since the cleavage site of aggrecanase on aggrecans is in their interglobular domain [48], so the fragment of the interglobular domain involving the cleavage site of aggrecanase is possible to be used as the sensor of an aggrecanase sensitive array to detect the activity level of aggrecanases with our methods in the future.
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