CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Surface-assisted Laser Desorption Ionization-Mass Spectrometry (SALDI-MS)

SALDI-MS is an analytical technique which can measure the masses of the atoms or molecules of sample. Mass spectrometry works by ionizing of chemical compounds to generate charged molecules or molecule fragments and measuring mass-to-charge ratios of sample. Ions usually have a charge of +1 ($Z = 1$), so the m/z equals the mass of the sample directly. The ionization and desorption processes out of the substrate, the atom is ionized by laser. In mass spectrometry, matrix is used to absorb the laser energy and transfer to analyte in ionization step. Matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) technique used organic matrix which can absorb laser energy and dissociate a proton, such as α-cyano-4-hydroxycinnamic acid (CCA) and 2,5-dihydroxybenzoic acid (DHB). These matrices can ionize and have interference peaks from ions generated from matrices at low mass region (m/z ≤ 500) in Figure 1(a). For SALDI-MS technique, a substrate is modified with matrix which does not ionize and disturbs analysis as shown in Figure 1(b). These substrates can be used for the analysis of low molecular weight sample. Moreover, samples for SALDI-MS analysis can be easily prepared by dropping directly sample onto substrate without having to mix sample with matrix. Analysis of samples having high concentration of salt can be done correctly both quantitatively and qualitatively.
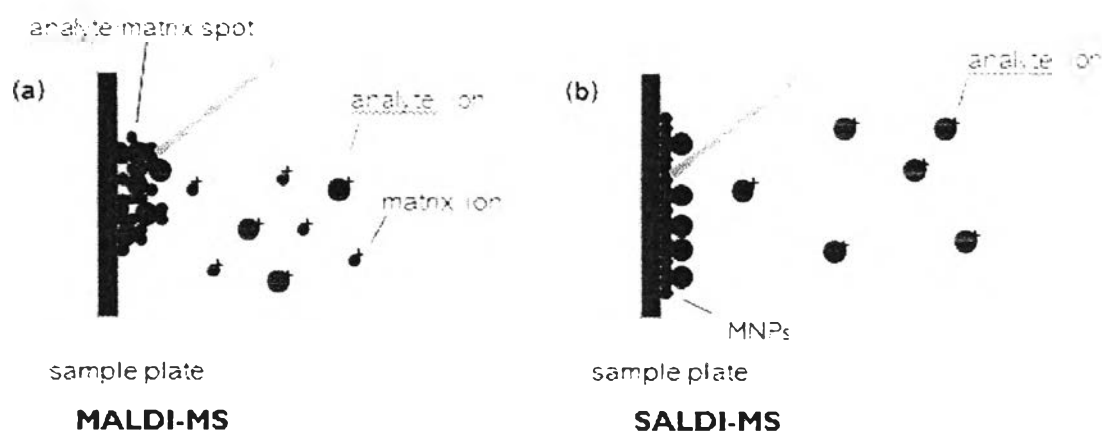
Figure 2.1 Ionization processes on the substrate of (a) MALDI-MS and (b) SALDI-MS

2.2 Patterning

Patterning provides high throughput analysis, also it is an important technique widely used in biological fields. Several techniques can be used to generate the pattern such as microcontact printing, microfluid network, spotting and photolithography. Among them, photolithography [12] scale is one of interesting technique used to prepare a small pattern in the micrometer on the surface, and ideal for creating a pattern on a silicon wafer for use in electronic technology. To prepare the pattern by photolithography, in the first step, light-sensitive chemicals (positive photoresist) are coated on the surface of silicon dioxide via spin coating. Then, photomask is placed on the photoresist-coated surface, and the surface is irradiated with UV light. After irradiating, the pattern is obtained after treating with solvent because oxide and photoresist layers are removed by etching process. For using negative photoresist, the crosslinked photoresist layer is formed by irradiating with UV light. Also, after exposing with solvent, the crosslinked photoresist layer is not removed whereas the area shield with photomask can be removed to generate the pattern. For the surface patterning, this work is interested in photolithography

method because it is rapid and easy technique. Small pattern with micrometer size scale can be prepared on the glass slide. Positive photoresist was selected for patterning. The positive photoresist does not crosslink after exposure to UV so that it can be removed to generate the area under photoresist for reacting in the next step of modification.

Many research works have reported on preparation of pattern via photolithography techniques.

In 2007, Dong *et al*. [13] prepared protein patterning via photolithography. Self-assembled monolayers of poly(ethylene glycol) (PEG) on silicon surfaces were patterned by coating positive photoresist. After a patterned PEGylated silicon surface was created, the etched regions were back-filled with a surface initiator for ATRP, and PAA brushes were grown, respectively. Moreover, avidin could be covalently attached to carboxyl groups in PAA brushes. For using biotin-tagged bovine serum albumin (BSA) as a model, the biotin-tagged protein could be bound through specific interactions, avidin-biotin interaction, while the PEG regions surrounding the PAA brush greatly reduced nonspecific adsorption.

In 2013, Jensen *et al*. [14] used an oxidative polymerization route to synthesize conjugated electroactive copolymer based on an alkoxy-substituted propylenedioxythiophene (ProDOT) and an acrylate-substituted ProDOT having the variation of monomer functionality with number average molecular weights (Mn) of 10-20 kDa. The synthesized polymer solutions showed excellent film forming by using both spray-casting and spin-coating. Utilizing photo-crosslinker, thin polymer films are insoluble in common organic solvents while maintaining electrochemical,

spectroscopic, and colorimetric properties. In addition, the applicability for patterning of electrochromic polymer film was studied by photolithographic methods and showed switch of pattern between a highly colored and near colorless transmissive state.

In 2011, Flavel *et al.* [15] developed a simple, photolithographic method for the fabrication of arrays of porous silicon spot by electrochemical anodization without the use of ceramic or metal assistive layers. The produced arrays of porous silicon were suitable for further chemical modification by hydrosilylation and then immobilization of the fluorescent dye lissamine and the arginine-glycine-aspartic acid-serine (RGDS) peptide to obtain photoluminescent and nonphotoluminescent porous silicon, respectively. The RGDS-functionalized surface provided the selective attachment of human lens epithelial cells on the patterns. This demonstrated the development of optical sensor arrays interfaced with mammalian cells in culture for nonintrusive detection of cellular processes.

In 2013, Zhou *et al.* [16] prepared pattern of poly(ethylene glycol) methacrylate–random-2-methacryloxyethyl trimethylammoniumchloride (poly (PEGMA-ran-MAETAC)) random copolymer brushes using surface-initiated atom transfer radical polymerization polymerization (SI-ATRP) reactions on silicon substrates via photolithography techniques to develop biocompatible polymeric materials for neuroprosthetic device coatings. The proposed method has been successfully used to develop biocompatible polymeric materials and effectively control growth of neurites for regeneration in the central nervous system.

In 2012, Goudar *et al.* [17] prepared protein microarrays on glass substrate. Firstly, they prepared the pattern via photolithography using a positive photoresist then silanised on photoresist patterned with 3-aminopropyltriethoxysilane (APTES), and thereafter reacted with proteins selectively for immobilized. In this research, they have successfully illustrated a simple process for fabricating high-density protein microarrays.
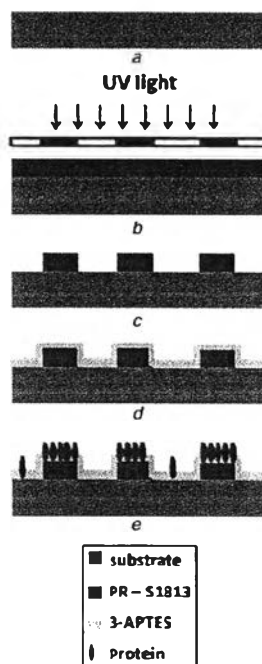

Figure 2.2 Schematic of the photoresist functionalisation method (a) piranha cleaned glass substrate, (b) UV exposure to the photoresist (PR) spun substrate, (c) patterns transferred to the photoresist, (d) silanising sample with APTES for 1 h, and (e) protein incubation. [17]

In 2013, Ren *et al.* [18] prepared micropatterning of single cell arrays on glass slide via photolithography for basic cell research and high throughout drug screening. Firstly, they prepared the pattern via photolithography using positive photoresist

then hexamethyldisilazane (HMDS) was deposited in the photoresist and residual photoresist was removed. PEG was immobilized at bare glass. They used hydrophobic HMDS islands for adhesion and hydrophilic poly(ethylene glycol) (PEG) regions as the passivator to improve the ratio of cells capturing. The biotin–(strept)Avidin System, the BSA and streptavidin, was used for studying cells capturing. The biotin–(strept)avidin system was attracted on the HMDS islands successively to form streptavidin arrays. They found that micropatterning of single cell arrays via photolithography can be prepared successfully and PEG regions greatly reduced nonspecific adsorption.
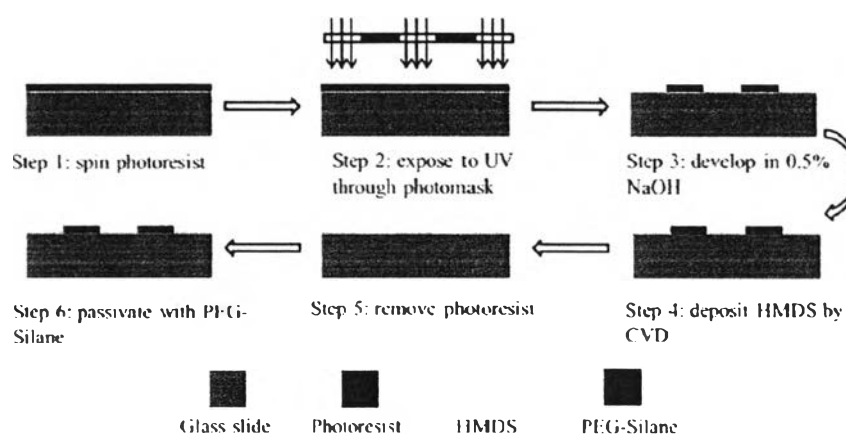

Figure 2.3 Diagram of the micropatterning process: (1) glass slides were spun with photoresist and soft baked, (2) photoresist was exposed with the photomask and post exposure baked, (3) photoresist was developed in 0.5% NaOH and hard baked, (4) HMDS was removed, and (5) the bare glass was passivated with PEG-silane. [18]

2.3 Polymer brushes

Polymer brushes are the polymer chains attached with one end to a surface via covalent bond, a much stronger linkage between polymer chains and substrate

than physical adsorption [19]. In general, the polymer brushes can be prepared by either "grafting to" or "grafting from" approaches. The grafting from method consists of polymer chain grown from surface attached with small initiators. Also, this method provides a very dense polymer brushes. For the "grafting to" method, polymer-grafted surface with sparse coverage is obtained as a result of an attachment of large polymer chain onto surface [20]. The synthesis of polymer chains has been performed using several techniques such as anionic, cationic, and free radical polymerization. The polymerization with well-controlled molecular weight and molecular weight distribution of polymer chain can be called controlled polymerization. Currently, popular "controlled" methods including reversible addition-fragmentation chain transfer (RAFT) polymerization [21] and atom-transfer radical polymerization (ATRP) [22, 23] are widely used.

This research will prepare polymer brushes of PAA via surface-initiated RAFT polymerization or "grafting from" method, with use of a dithioester chain transfer agent. RAFT is an attractive method to synthesize polymer molecules having well-controlled molecular weight. Moreover, RAFT polymerization provides many advantages: applicability for various water-soluble monomers, lack of metal catalyst use which is desirable for using in bio-application.

A number of research work reported on synthesis of PAA via ATRP and RAFT polymerization:

In 2011, Akkahat *et al.* [24] prepared surface-tethered PAA brushes for using as a 3D sensing platform for the specific biomolecule immobilization and detection. In this research, poly(tert-butyl acrylate) (Pt-BA) brushes was firstly prepared by SI-

ATRP, and then the tert-butyl groups from the Pt-BA brushes were removed by acid hydrolysis to obtain PAA brushes. The ability for using PAA brushes as a three dimensional (3D) precursor layer for biosensing applications were investigated by surface plasmon resonance (SPR). From SPR results, the biotin-attached PAA brushes showed high signal for the specific binding of streptavidin in comparison with a self-assembled monolayer (SAM) of a carboxyl-terminated alkanethiol, used as a model two-dimensional (2D) conventional precursor layer. Moreover, The PAA brushes showed very low non-specific adsorption with BSA and streptavidin.

In 2009, Ji *et al.* [25] synthesized high molecular weight poly(acrylic acid) (PAA) directly in aqueous solution via RAFT polymerization by using a water-soluble trithiocarbonate, 2-[[(ethylsulfanyl)carbonothioyl] sulfanyl]butyric acid, as a RAFT agent. The ratios between AA monomer and RAFT agent ([AA]:[RAFT]) were varied to obtain the different molecular weights. At the different [AA]:[RAFT] ratios showed linear increase of molecular weight and low PDI. These results indicated that the RAFT polymerization of PAA in aqueous solution was well-controlled.

In 2013, Qu *et al.* [26] prepared PAA brushes on the surface of silica nanoparticles (SiO_2) by RAFT polymerization. A silane functionalized RAFT chain transfer agent was firstly synthesized and immobilized on silica nanoparticles (SiO_2-RAFT). Then, PAA brushes were polymerized from SiO_2-RAFT to obtain SiO_2-PAA nanoparticles. The particles were characterized by transmission electron microscopy, dynamic light scattering, gel permeation chromatography, thermogravimetric analysis and conductometric titration. The SiO_2-PAA nanoparticles have a brush thickness of 14.6–68.8 nm, grafting density of 0.23–0.34 nm with a carboxyl group content of

0.82–2.37 mmol/g. In addition, the protein binding capacity to the brushes was improved to 2600 µg streptavidin/mg due to its high carboxyl content and the spherical brush structure.

Many research works has been reported on substrate modification to be used for SALDI-MS technique:

In 2008, Chiu *et al.* [27] have reported the immobilization of PAA (MW ~ 2 kDa) brushes onto the surface of the iron oxide particles. PAA contains a number of carboxylate groups which are capable of chelating with iron oxide nanoparticles and acting as proton source for protonation of analyte. The modified particles can be used as matrix for SALDI-MS analysis without the addition of extra proton source. Biomolecules, namely bradykinin, mellitin, and insulin were used as samples for feasibility test. The upper detectable mass limit in this research is ~6 kDa.

In 2008, Shrivas *et al.* [10] analyzed sulfur drugs and biothiols using silver nanoparticles (AgNPs) capped with different functional groups as the matrix and affinity probes which can bind with thiol (-SH) groups of analyte. The proposed method has been successfully applied for the determination of cysteine and homocysteine in human urine samples using an internal standard. The limit of detection (LOD) and limit of quantification (LOQ) for cysteine and homocysteine in urine sample were 7 and 22 nM, respectively.

In 2010, Chiang *et al.* [9] have investigated six nanomaterials (AuNPs, TiO_2 NPs, Se NPs, CdTe quantum dots (QDs), Fe_3O_4 NPs, and Pt nanosponges (NSPs)) for their applicability as surfaces for the analyses of peptides and proteins using SALDI-MS. They found that nanomaterial has several advantages over organic matrices. Different

NPs were tested with a range of molecular weight of analytes. AuNPs provided the best sensitivity for small analytes; Pt NSPs and Fe_3O_4 NPs provided the highest mass limit (25 kDa) for proteins.

In 2009, Tarui *et al.* [28] developed substrate for SALDI-MS analysis using nanocomposite films of cationic diblock copolymer micelles [poly(styrene-b-N-methyl-4-vinyl pyridiniumiodide)] and ammonium citrate-stabilized Au nanoparticles on silicon. They found that the substrate based on nanocomposite films provided high ionization efficiency. Moreover, clean spectrum with low matrix background from Au clusters was obtained due to good distribution of NPs within the polymer film.

In 2011, Aminlashgari *et al.* [29] utilized nanocomposite films as easy-to-handle surfaces for SALDI-MS analysis. They immobilized the particles in the polymer matrix, polylactide (PLA). They used several types of NPs such as TiO_2, MgO, SiO_2 etc as matrices for analysis of small molecules, acebutolol, propranolol and carbamazepine. They found that the advantages of nanocomposite films compared to the free nanoparticles used in earlier studies are the ease of handling and reduction of instrument contamination because the particles were immobilized into the polymer matrix.

In addition, there are researches related to *in situ* synthesis of NPs within polymer matrix.

In 2003, Boyes *et al.* [11] synthesized diblock copolymer of polyelectrolyte brushes of either polystyrene (PS) or poly(methyl acrylate) (PMA) and poly(acrylic acid) (PAA) on silicon surfaces. Diblock copolymer brushes were synthesized using

sequential ATRP from the self-assembled monolayer of a bromoisobutyrate initiator. They found that the polyelectrolyte diblock copolymer brushes can be used for the synthesis of inorganic nanoparticles, AgNPs and PdNPs, by reduction of Ag^+ and Pd^+, respectively by the treated PAA.

In 2007, Bai *et al.* [30] successfully prepared AuNPs dispersed in polyacrylamide (PAM) matrix by facile and simple method. The AuNPs/PAM nanocomposites were obtained by reduction of gold salt with aqueous PAM solution and stabilization by PAM immediately indicating that PAM acts as both stabilizer and reducing reagent without adding of external reducing agent. The nanocomposites were characterized by X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), and ultraviolet–visible (UV–vis) absorption. From the FTIR, it was found that the frequency of the carbonyl group stretching in nanocomposite was shifted from 1676 cm^{-1} to 1632 cm^{-1} demonstrating that PAM molecules absorb on the gold nanoparticles surface via coordination bond between gold atom and oxygen atom of the carbonyl groups in PAM.

In 2012, Woo *et al.* [31] improved the efficiency of organic solar cells via the incorporation of gold (Au) or silver (Ag) nanoparticles (NPs) in the hole-transporting buffer layer of poly(3,4ethylenedioxythiophene):poly(styrenesulfonate) (PEDOT:PSS). The composite materials were successfully synthesized by a simple *in situ* preparation method which involved the reduction of chloroauric acid ($HAuCl_4$) or silver nitrate ($AgNO_3$) with sodium borohydride ($NaBH_4$) and showed good dispersion of NPs in the PEDOT:PSS media.

From past researches, it was found that NPs can be used to improve the surface to enhance analysis by SALDI-MS techniques. However, to date there has been no report on the use of polymer brushes to synthesize NPs as matrix for SALDI-MS. In this research, we are interested to develop a substrate for separation and analysis of peptides by SALDI-MS. The patterned PAA brushes were prepared on glass substrate via photolithography and RAFT polymerization. The carboxyl groups of PAA brushes act as reducing moieties for *in situ* synthesis of AuNPs, without the use of additional reducing agent. Moreover, carboxyl groups of PAA may serve as internal proton source. It is anticipated that this PAA brushes substrate containing AuNPs can be used for pre-concentration and separation of thiol-containing peptide from the peptide mixture in SALDI-MS analysis.