

Chapter 8

Inkjet Printing of Biomolecules for Biorecognition

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8.1 Introduction of Inkjet Printing Technology

8.1.1 Background

Inkjet printing is undoubtedly the most familiar and most widely applied technology for personal office desktop printing nowadays. It can be stated without exaggerating that the arrival of this technology has revolutionized the possibilities of desktop printing in many aspects, but in particular when it comes to color printing. In contrast to the previously dominant desktop printing technologies (e.g., ink ribbon-based dot-matrix printing), inkjet printing made it possible not only to print simple text but also to instantly reproduce digital color graphics and photographs in astonishingly high quality. Before the implementation of personal desktop inkjet printers, color printing of similarly high resolution was to a large extent unaffordable for the common user. The benefits of inkjet printers, that we take for granted in our daily use nowadays, are the result of a continuous technical development, which has started in 1949 with the filing of the first patent application regarding a practical inkjet device [1]. However, several decades of further research and development were necessary, before the first successful and affordable mass-marketed desktop inkjet printer became available from Hewlett-Packard (HP) in 1984 [2].

In terms of technical functionality, an inkjet printer is straightforwardly simple. It is an electronic device enabling the direct deposition of very small droplets of liquids (inks) onto a well-defined and user-controlled position of a substrate. As implied by the name of the technology, the ink droplets are ejected (jetted) from a small nozzle of the printhead onto a specifically determined position of the substrate. In contrast to several other deposition methods for liquid droplets, this process is performed without any physical contact between the printhead and the substrate.

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Therefore, inkjet printing is referred to as a noncontact liquid dispensing method. Furthermore, compared with most other major printing techniques currently in use (e.g., flexography, gravure printing, offset printing, screen printing), the inkjet printing process is fully digitally controlled, and allows for the “direct transfer” of electronically stored information onto a print substrate. It does not require the costly and time-consuming conversion of electronically stored data into printing plates or screens, before transferring it to the substrate. On the other hand, a graphical image printed by inkjet technology is made up of large numbers of individually deposited ink droplets. Similar to the case of the former ink ribbon-based dot-matrix printers, such a “dot-by-dot” image buildup process is more time consuming compared to plate- or screen-based ink deposition, where a complete image can be transferred to the substrate in a single step. As a consequence, inkjet printing can currently not and will probably never compete with other major printing methods in terms of speed when it comes to the reproduction of large numbers of identical prints. But its digital feature with the possibility to directly transfer an image onto a substrate allows for a very high degree of flexibility. This is particularly advantageous in printing of multiple items in lower number of replicates, in industrial prototyping applications, or for marking and coding of objects with continuously changing patterns [3].

Although most readers will immediately associate the inkjet technology with personal office use, industry-related applications actually go further back in history than the currently best-known and widely spread desktop printing. The technology was first routinely used for marking and coding of products, in what Martin and co-workers refer to as the first generation of commercial inkjet applications [3]. Since every pattern or code deposited on a product or package is unique, inkjet printing without the need for printing plates is advantageous compared to other printing methods. In addition, the noncontact characteristics allow for larger distances between printhead and print substrates which is useful in the setup of industrial production lines. Personal office desktop printers are regarded as the second generation of commercial inkjet applications. Their popularity in relation to cost/performance ratio is currently unbeaten. Only more recently, the enormous potential of inkjet printing has been discovered for industrial applications going beyond the reproduction of text and images on paper, films, or other substrates in what is now referred to as the third generation of commercial inkjet applications. The technology has evolved into full-scale industrial mass production. A popular example is the fabrication of color filters for light emitting diodes and large flat-panel color displays [4]. Another relatively recent but rapidly growing addition to the fields of application for inkjet printing technology is the domain of printed electronics [5].

In this chapter, however, focus is set on a different but equally promising and comparably new application of inkjet printing technology, the printing of biomolecules. In this case, inkjet printers with the ability to reproducibly deposit known and small volumes of liquids onto specific user selectable spots on a substrate can be regarded as accurate tools for liquid dispensing, which are highly important in the fields of chemistry, biology, or life sciences. In this context, inkjet printing is competing with alternative liquid dispensing methods, such as pipetting, contact printing, and pneumatic dispensing, among others [6].

In the following section, the basic mechanic principles of the most commonly applied inkjet printing methods are shortly introduced. This is important in order to understand not only the great potential but also the challenges faced when using inkjet printing as a liquid dispensing method for the deposition of biomolecules on a substrate targeting selective biorecognition.

8.1.2 Technical Principles of Inkjet Printing

Commonly applied inkjet printing technologies are divided into two main categories known as “continuous inkjet printing” (CIJP) and “drop-on-demand” (DOD) inkjet printing (Fig. 8.1). Historically, continuous inkjet technology is the older of the two. Its basic working principle is schematically illustrated in Fig. 8.2: a jet of ink liquid ejected from the printhead nozzle by pressure is broken-up into a stream of small droplets by application of a mechanical disturbance pattern of a specific frequency (e.g., by vibration of a piezoelectric actuator). Selected drops within this stream are subsequently electrically charged while passing through an electrostatic field between two charging electrodes. Further along the drops’ flight path is a second set of electrodes with an applied high voltage electric field, resulting in the selective deflection of electrically charged drops only. In most printing systems, the deflected charged drops are guided towards the printing substrate, whereas the uncharged drops fly straight into an ink collection system for reuse. In the simplest practical implementation of this technology, electronically stored digital data is converted into graphical data on the substrate by switching between charged and uncharged ink drops. In more advanced systems, varying the charge of the drops

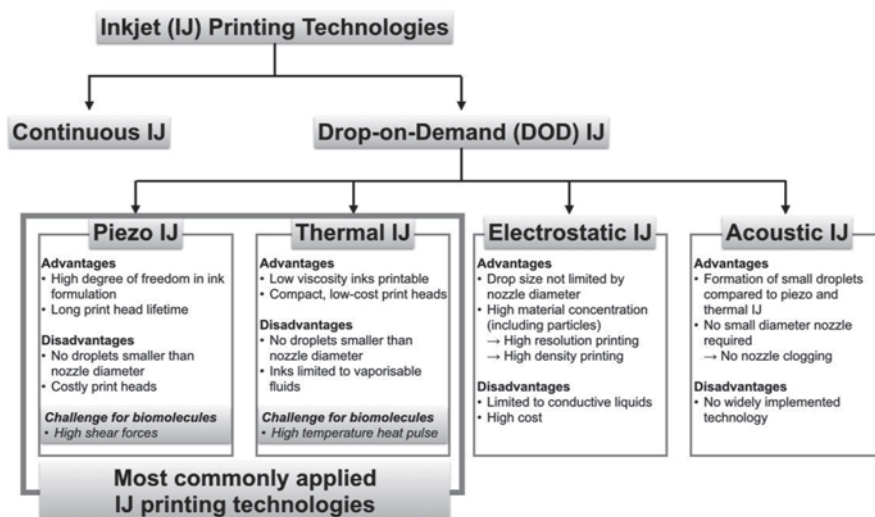


Fig. 8.1 Overview of major inkjet printing technologies

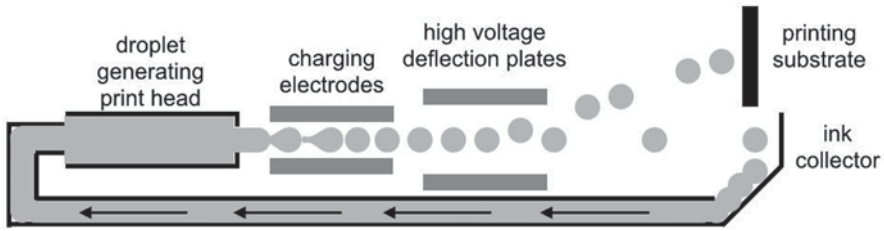


Fig. 8.2 Schematic representation of the continuous inkjet printing principle

results in different deflection angles and correspondingly in different places of drop impact on the substrate. The continuous inkjet printing mechanism requires the created drops to become electrically charged and therefore, poses restrictions on the choice of printable fluids. In addition, the achievable printing resolution is comparably low. An advantage of the technology is the possibility of achieving high drop firing frequencies (typically around 80–100 kHz, but reaching values up to 1 MHz) [7], which result in comparably high printing speeds, and high drop velocities (typically around 20 m/s) [8]. These characteristics allow for larger distances between the printhead and the substrate [9].

The newer and currently most widely applied principle in inkjet printing is the drop-on-demand (DOD) technology. In contrast to the previously described continuous inkjet approach, where a continuous stream of drops is digitally controlled to split into drops reaching the substrate and drops being recycled, DOD printing is based on the control of the drop formation itself. As indicated by its name, drops of ink are ejected from the printhead nozzle only on demand. The first big advantage of this method is the absence of the necessity to create electrically charged ink droplets that strongly expands the choice of potential ink fluids. The second significant advantage is the elimination of the ink recycling system, since all of the created ink droplets are targeted at the substrate. A drawback in comparison with continuous inkjet printing is the significantly lower maximum achievable drop firing frequency of 0–25 kHz [10], with maximum values up to only 100 kHz [8], resulting in reduced printing speeds. The reason for this difference is the fact that a single cycle consisting of drop ejection and subsequent ink refill of the firing chamber has to be completed before the process can be repeated. Furthermore, typical drop velocities for DOD inkjet printing are in the order of 10 m/s which is lower than in the case of continuous inkjet printing. As shown in Fig. 8.1, DOD inkjet printing technologies are commonly further subdivided into four major subgroups (piezoelectric, thermal, electrostatic, and acoustic inkjet printing), named according to the mechanisms used for droplet formation. In the following, all of the four mechanisms are shortly introduced for the sake of completeness. However, focus is set on piezoelectric and thermal inkjet printing, since those two technologies are by far the most technically matured and dominant in current applications. Table 8.1 summarizes major characteristics and physical parameters associated with these two important inkjet printing technologies. Generally, piezoelectric technology is dominating in the industrially related inkjet printing market, whereas thermal inkjet printing has become the major

Table 8.1 Typical values/ranges of physical parameters for piezoelectrically and thermally actuated DOD inkjet printing

Parameter ^a	Piezoelectric IJ	Thermal IJ	References
Nozzle diameter (μm)	20–30		[14]
Drop diameter in flight (μm)	15–55		[8]
Drop volume (pL)	Typically 10–20, minimal 1, maximal 5000		[10, 14]
Drop velocity (m/s)	3–15		[8]
Firing frequency (kHz)	Maximum values up to 100, commonly 0–25		[8,10]
Ink viscosity (cP)	Minimal 5–10 typically < 20	Minimal 1–1.5 typically < 3	[15–17]
Ink surface tension (mN/m)	Typically 28–40		[18]
Shear rate (s^{-1})	2×10^4 – 2×10^6	10^4	[11, 10]

^a The listed values and ranges are significantly varying depending on the literature references and on the printing equipment applied. Therefore, they should be regarded as guidelines, rather than strict requirements

technology in consumer desktop printing. In the inkjet-based deposition of biomolecules, DOD piezoelectric and thermal inkjet printing are currently the only routinely applied technologies, with the piezoelectric approach leading in terms of number of application examples. One reason for this bias towards piezoelectric methods is probably simply their dominance in the industrial inkjet printing sector, although other factors eventually play a role, as will be shown later on in this chapter. On the other hand, no reports on biomolecule-related applications of continuous inkjet printing can be found in the literature, most probably because of the significantly less economical use of ink.

8.1.2.1 Piezoelectric Inkjet Printing

In the piezoelectric inkjet printing mechanism, a drop of ink is ejected from the printhead nozzle by fast and reversible deformation of the firing chamber (Fig. 8.3a). This deformation on a sub-micrometer scale is achieved by applying an electronic signal (voltage pulse) on a piezoelectric actuator surrounding the firing chamber, resulting in a pressure wave propagating towards the nozzle at the chamber exit. The shape of the voltage pulse, characterized by its slope (voltage increase with time), amplitude (maximum voltage), and length, allows to control the ink ejection process. Depending on ink parameters, such as viscosity and surface tension, different pulse shapes are required. However, in general, piezoelectric inkjet printing is characterized by a high degree of freedom in ink formulation. The possibility of having multiple voltage pulse parameters to adapt besides the physical properties of the ink is certainly a big advantage of the technology. A drawback is the fact

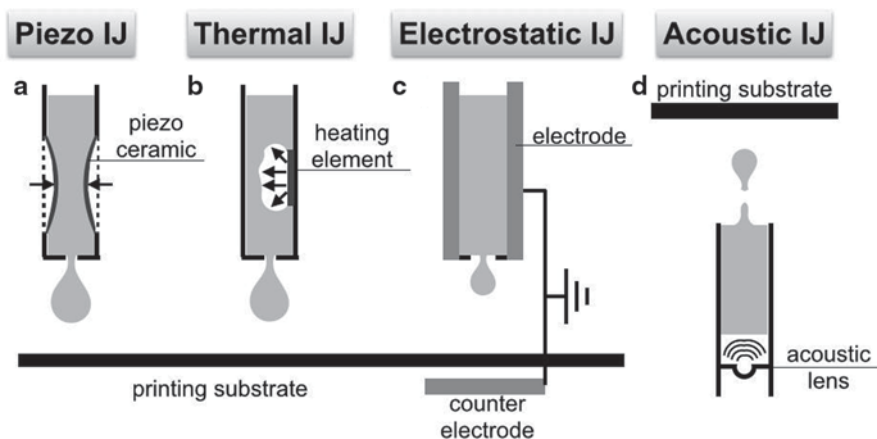


Fig. 8.3 Schematic representation of the major drop-on-demand (DOD) inkjet printing technologies

that the minimally achievable drop size is limited by the diameter of the printhead nozzle. A factor that has to be considered in the case of working with ink compositions containing biomolecules are the significant shear rates that are experienced by the liquid (Table 8.1). Although shear stress is not unique to piezoelectric inkjet printing and occurs in all jetting methods with sudden mechanical stress applied to the ink liquid, it tends to be higher compared to other printing technologies. The reason for this is the fact that piezoelectric printing generally requires liquids of a higher viscosity (Table 8.1) compared with, for example, the thermal inkjet printing method described in the next section.

8.1.2.2 Thermal Inkjet Printing

In thermal inkjet printing, drop generation in the firing chamber occurs by the creation of a rapidly expanding vapor bubble, which results in the displacement of ink from the nozzle (Fig. 8.3b). The bubble is created by local heating and vaporization of ink by means of a heating element located inside the printhead's firing chamber. Upon elimination of the heat pulse after drop ejection, the vapor bubble rapidly collapses creating a vacuum that is filled by drawing in ink from the ink reservoir, after which the system is ready for the next firing cycle. The requirement to locally vaporize the ink fluid poses restrictions on the applicable fluids. Water is the material of choice for the formulation of thermal inkjet printing ink compositions. Generally, a heat pulse in the order of typically around 200–300 °C is applied. At first sight, this high temperature pulse might certainly be regarded as detrimental for the use in combination with comparably heat-sensitive biomolecules. However, it should be noted that the duration of this pulse is in the order of around 2 μ s [11]. In addition,

a high gradient of decreasing temperature from the surface of the heating element into the bulk of ink liquid is assumed, so that in fact the temperature experienced by the majority of biomolecules in the liquid volume is far below the 200–300 °C applied for the heat pulse. In fact, it has been stated that only about 1 % of the ink volume of the firing chamber is exposed to high temperature, whereas the bulk of the fluid remains at merely around 10 °C above ambient temperature [12]. The drop ejection method applied in thermal inkjet printing technology allows to work with ink liquids of very low viscosities (Table 8.1) that results in lower shear rates experienced in the ink liquid. As in the case of piezoelectric inkjet printing, the minimally achievable drop size for thermal inkjet printing is equally limited by the diameter of the printhead nozzle.

8.1.2.3 Electrostatic Inkjet Printing

In this technology, a voltage is applied between the printhead nozzle and an electrode located behind the printing substrate (Fig. 8.3c). Droplets are created by pulling out the liquid under the influence of the resulting electrostatic field. A great advantage of the method is the possibility to create liquid droplets that are smaller than the aperture of the printhead nozzle which is generally not possible in the case of piezoelectrically or thermally actuated drop generation. This feature is potentially of high interest when working with liquids containing solid particles or relatively high concentrations of dissolved materials. The option to create small droplets, but at the same time being able to use printhead nozzles with sufficiently large diameter to prevent particle-induced clogging, is of high relevance. However, the limitation to the use of conductive liquids as ink carriers is a disadvantage preventing the more widespread development and use of the technology. As a consequence, the costs for implementing electrostatic inkjet printing methods are currently comparably high.

8.1.2.4 Acoustic Inkjet Printing

In this version of inkjet printing technology, liquid drops are generated by the use of focused, high-intensity sound beams directed to free liquid surfaces through an acoustic lens (Fig. 8.3d). The sound waves traveling towards the liquid–air interface cause the formation of a mound of liquid and ultimately a droplet breaking free. In principle, this method does not require an actual nozzle and therefore, features similar advantages to electrostatic inkjet printing in the sense that there is no need to worry about printhead nozzle clogging even when using concentrated ink liquids or inks with a high content of solid particles. At the same time, the diameters of ejected drops can be as small as 5 μm [13] which can neither be achieved by piezoelectric nor thermal inkjet printing. Unfortunately, however, acoustic inkjet printing technology currently remains a minor technology.

8.2 General Considerations for Inkjet Printing of Biomolecules

It is important to keep in mind that inkjet printing technology has not been specifically developed for the application to biomolecules such as nucleic acids and proteins. When compared to constituents of inks for ordinary graphical printing applications (organic and inorganic pigments, organic dyes, etc.), biomolecules are of much more fragile nature. The preservation of their structure and function is generally dependent on several environmental factors such as temperature, solvent, and pH. In this context, the abovementioned high temperature heat pulse applied for drop ejection in thermal inkjet printing is, at first sight, recognized as the most obvious major concern. However, in addition, most biomolecules can be regarded as relatively large macromolecular compounds, in contrast to low-molecular weight colorants used in graphical printing inks. The mechanical forces encountered in both piezoelectric and thermal inkjet printing (e.g., shear forces, high acceleration rates, ink compression upon ejection, high speed droplet impact on the printing substrate, etc.), all have sufficient potential to break or deform larger 3D molecular structures common for biomolecules. For this reason, there is no “safe-zone” in terms of choice of inkjet printing technology (piezoelectric or thermal). As shown in the following example of selected applications, an experimental evaluation is required from case to case. The trend for piezoelectric actuation to dominate the field of bio-ink printing is probably more historically related than caused by biomolecule stability issues.

It is also not possible to define a list of biomolecules suitable or unsuitable for deposition by inkjet printing. In all cases, it will be the combination of three major factors responsible for success or failure of the inkjet printing approach: (1) selection of the printing technology and droplet ejection pulse parameters; (2) choice of ink solvent and additives; and (3) printing substrate. Among those three factors, the ink composition probably offers most options for adaptation and optimization, but at the same time can also be a major source of trouble. As shown in the following sections, certain ink additives can have a positive influence on the stability of biomolecules during the printing process. However, their effect on the printing performance in terms of reliability and reproducibility is not always positive and has therefore to be individually evaluated. In many cases, compromises between biomolecule solubility and stability as well as ink jettability have to be made. In terms of ink solvent, water (mostly pH buffered) is the most obvious choice when working with biomolecules. Interestingly, although its use is less often reported for the deposition of biomolecules, thermal inkjet printing technology has, from its beginning, been much more suitable to handle purely aqueous liquids, compared with piezoelectrically actuated printing. Actually, many industrial-use piezoelectric printheads have been originally incompatible with water-based ink formulations, although this deficiency is now mostly overcome driven by market demands [9].

The printing substrate also plays an essential role in the overall printing process, not only in relation to the more obvious print resolution and quality but also to biomolecule stability. The influence on resolution and quality is not limited to working

with biomolecules, but is common to all inkjet printing processes in general. Print resolution and quality are not determined by the ink droplet size alone. They depend, to a large extent, on the spreading of the ink after drop impact on the substrate, which is primarily determined by the substrate surface energy in relation to the surface tension of the ink. Other relevant parameters include the liquid penetration into or adsorption onto the substrate and the evaporation rate of the ink solvent. The latter, on the other hand, depends on environmental factors such as temperature and humidity. More limited to the inkjet printing of biomolecules is the influence of the print substrate on the biomolecule activity. Although primarily evaluated in relation to the inkjet deposition of living cells, it has been shown that softer and more viscous substrate surfaces can reduce the impact forces acting on droplets, however, at the cost of decreasing resolution [12].

One of the motivations to use inkjet printing for the deposition of biomolecules onto a solid substrate is the ability to achieve high patterning densities (e.g., for DNA microarrays). With presently commercially accessible standard technology (excluding customized systems), droplet sizes down to 1 pL are becoming routinely achievable for optimized ink compositions. In combination with an optimized printing substrate and very precise substrate positioning control, this translates into reproducibly deposited spots of about 30 μm diameter under standard operation conditions, with sizes as low as 10 μm for highly controlled laboratory environments [9]. In the case of using self-aligned printing approaches that rely on selective de-wetting of low surface energy areas, features of below 100 nm have been achieved by inkjet printing alone [19]. However, this has been successfully demonstrated for the printing of electronic features, it has to the best of our knowledge not been realized for biomolecules.

Another point to consider is the selection of the inkjet printer for the deposition of biomolecules. The first and biggest distinction is between the affordable consumer desktop printers and the much more expensive research-use inkjet material printers specially dedicated to non-graphical printing applications. When it comes to thermal actuation, consumer desktop printers are currently the only option. At present, Canon and HP are the dominating manufacturers of thermal inkjet printers. In the case of piezoelectrically operated desktop printers, Epson and Brother are probably the biggest players in the global market at this time. One significant disadvantage when working with desktop printers in terms of convenience is the fact that, depending on the country, empty (virgin) ink cartridges are often not easily available. Therefore, the user is required to disassemble and clean the originally provided ink tanks, before being able to work with self-made bio-inks. A further potential disadvantage of desktop printers is the substrate feeding mechanism which is primarily designed for handling paper. Difficulties can occur with thicker and less flexible materials, unless a printer with a direct CD/DVD print option is used. In addition, it is probably needless to say that the degree of freedom and the reproducibility of substrate positioning can easily become an issue, the latter in particular if multiple print runs are required. Finally, all desktop printers are essentially “black box” systems with limited user control. A big advantage of all consumer desktop printers is that they come equipped with at least four ink tanks (black, cyan, yellow, magenta), thus theoretically allowing the simultaneous use of multiple inks [6].

Inkjet printing systems dedicated to research laboratory use are generally operating with piezoelectric actuation. In contrast to the consumer desktop printers, they offer a high degree of user-controllable features such as a choice of printhead nozzle diameters, the possibility to heat nozzle and substrate stage, visual confirmation of ink droplet ejection, and control of piezo-pulse voltage and duration.

With any type of inkjet printing equipment, there is always a risk of clogging the small printhead nozzle apertures. For this reason, an inkjet printing ink should always be passed through a filter with pore sizes below 1 μm (ideally around 200 nm) before filling a printer ink tank. As long as all used compounds are of sufficient solubility in the chosen ink system, the risk can be minimized. However, in the case of formation of precipitates or aggregates, clogging of the printhead is highly likely. In particular when working with inks containing particulate matter, dispersion stability is a very important factor. As a general “rule of thumb,” it is advised to limit the size of particles in inkjet printing inks to a value not exceeding 1/100 times the diameter of the chosen printhead nozzle [15]. This generally corresponds to particle sizes of ≤ 300 nm.

8.3 Inkjet Printing of Nucleic Acids

8.3.1 Introduction

Nucleic acids (DNA, RNA) play vital roles in the conservation of gene information and protein synthesis. Since the discovery of the double helix structure of DNA by Watson and Crick, DNA sequencing has attracted much attention as a fundamental approach for the analysis of specific gene information. Especially the comparative study of DNA sequences is motivated by multiple objectives (e.g., elucidation of the function of specific genes, disease-associated gene expression). In addition to DNA sequencing, the quantification of nucleic acids in biological samples is a valuable analytical approach providing information on gene expression levels. Simultaneous detection and quantification of multiple nucleic acids is of high interest for example for the analysis of gene mutations, the study of gene expression levels in cells, and its application to drug development, among others. In order to detect an exhausting number of DNAs in a single experiment, DNA arrays have been prevalently employed since the late 1980s. This very important type of selective biorecognition platforms is manufactured by immobilizing various single strand DNA sequence probes onto a solid support to capture and detect target DNAs by employing the well-known preferential binding between complementary DNA single strands. To obtain high-density DNA arrays, several technologies to deliver small amounts of probes onto a substrate have been developed so far [20–24]. Currently, photolithography [25, 26], pin spotting [27], and inkjet printing [28, 29] are representative methods applied in commercial manufacturing, with other types of deposition techniques being reported (maskless photolithography [30], electronic addressing

[31, 32], etc.). Among them, owing to its noncontact, fine, and precise patterning capability, inkjet printing technology is applied for the deposition of DNA on solid supports via two strategies: direct spotting of DNA and in situ synthesis of oligonucleotides on the solid substrate. In this section, the inkjet printing of nucleic acids is mainly described from the point of view of DNA microarray fabrication.

8.3.2 Common Microarraying Technologies

As mentioned above, several methods exist to deposit nucleic acids on a solid support for preparing DNA arrays. Primary approaches and their features are summarized in Table 8.2. The combination of photolithography and solid-phase synthesis of oligonucleotides was first introduced by Affymetrix [25] and has become one of

Table 8.2 Major approaches to nucleic acid microarraying

Arraying technology	Applicable materials	Substrate contact	Advantages	Disadvantages
Photolithography [25, 26]	DNA nucleotides	No	Achievable high density	Requirement of UV source and specific photomasks Risk of straying UV irradiation to nearby areas Limitation of oligonucleotide length (ca. 25-mer)
Maskless photolithography [30]	DNA nucleotides	No	Achievable high density Flexible sequence design	Requirement of UV source and sophisticated instruments Limitation of oligonucleotide length (ca. 25-mer)
Pin spotting [27]	Complete DNA	Yes	Low fabrication cost per spot	High risk of contamination of the printing device Damage of the spotter in every printing cycle
Inkjet printing [28, 29]	Complete DNA DNA nucleotides	No	Flexible sequence design Low risk of contamination	Difficult to clean the nozzle completely Risk of nozzle clogging
Electro addressing [31]	Complete DNA DNA nucleotides	No	Fast hybridization time Controllable hybridization stringency	Employment of sophisticated semiconductor devices Requirement of labeling of substrate and probes

the most familiar arraying techniques. It involves the delivery of DNA nucleotides (monomers) protected by photochemically cleavable groups, followed by local UV illumination through photomasks for deprotection. In this approach, 4^n different sequences of n -mer oligonucleotides can be created by $4n$ chemical cycles. The method provides high-density arrays (400,000 probes on 1.6 cm^2) [26], but can be time consuming to obtain long oligonucleotides, because of its serial procedure. In addition, it is comparably costly to design new sequences owing to the requirement of the corresponding number of different photomasks. On the other hand, an alternative photopolymerization approach has been developed by a group at the University of Wisconsin, where a computer connected to a digital micromirror array was employed to control the reflection of UV light from the light source to replace the photomasks [30].

A Stanford University group first demonstrated the deposition of pre-synthesized DNA onto a solid support by contact printing methods [27]. The contact dispensing systems involve the direct contact between the substrate and the ink-loaded dispenser such as microspotting pins, tweezers, and microstamps [22]. Their simple principles based on reciprocating movement of the dispenser between sample reservoir and printing matrix yield reproducible results with comparably little maintenance. Furthermore, the length of nucleic acids to be delivered is not limited. However, the limited fabrication throughput, the achievable density, and the fear of contamination through direct contact of dispenser and substrate are potentially problematic.

In contrast, inkjet printing technology is prevalently utilized as the most common approach in noncontact dispensing. As outlined before, inkjet printing technology allows handling of very tiny droplets of as small as nanoliter to picoliter volumes with good accuracy, and thus, is ideal for obtaining quantitative, high-density, reproducible arrays. Although inkjet printing has shortages in terms of the restricted choice of “ink” compositions and durability of the dispenser (i.e., print head), the fine tunability of droplet volumes by the applied voltage (piezoelectric actuation) or temperature gradient (thermal actuation) and the achievable high resolution features have motivated academic research and commercialization. Furthermore, the flexibility in spatial control significantly facilitates the creation of different DNA sequences at each spot, and leads to the preparation of arbitrarily designed DNA microarrays. As a consequence, Agilent has adopted this technology for manufacturing both custom and catalogue-order arrays.

8.3.3 Inkjet Printing of Nucleic Acid Arrays

Inkjet printing technology enables two approaches for arraying nucleic acids on a solid support (typically glass slide, silicon wafer): (1) printing of complete DNAs (pre-synthesized or PCR amplified), and (2) in situ synthesis of oligonucleotides by delivering DNA nucleotides (monomers) (Fig. 8.4).

The feasibility of inkjet printing of pre-synthesized or preliminarily PCR-amplified DNA sequences has been first demonstrated in the 1990s. In one of the earlier reports, Schober et al. employed a piezoelectric inkjet dispenser (Microdrop,

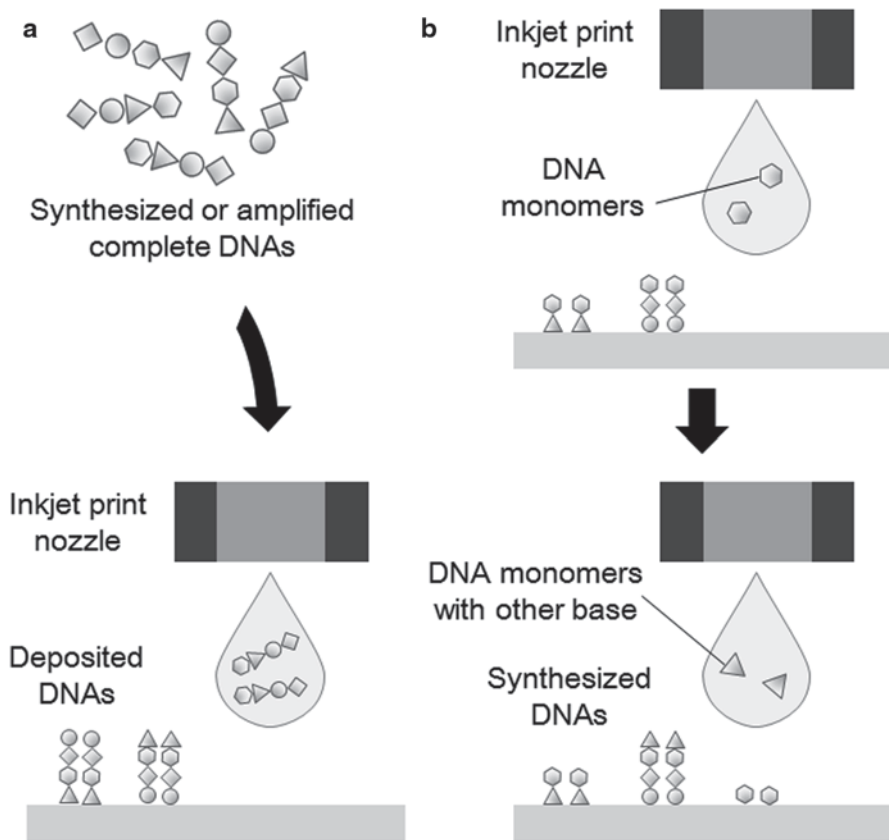


Fig. 8.4 Two approaches to obtain DNAs on a solid substrate: **a** deposition of complete DNA, **b** in situ synthesis by DNA nucleotide (monomer) printing

Norderstedt, Germany) as an alternative to pipetting small volumes of biological samples with high speed and high accuracy in 1993 [33]. They reproducibly handled volumes from a few nanoliters to as small as 5 pL with high frequency (10,000 drops per second), which surpassed piston-operated pumps, the conventional dispensing systems for biological samples at the time. By using this piezoelectrically actuated system, primary biological samples including DNA and RNA, and even enzymes and cells were successfully dispensed. Surprisingly, in spite of the high acceleration (100,000 g) encountered during the ejection process, no differences were confirmed in gel electrophoresis performed for RNA and DNA samples before and after being subject to the printing process. In 2000, Okamoto et al. first succeeded in printing a microarray of pre-synthesized 18-mer oligonucleotides onto a glass surface by using a modified personal desktop Canon Bubble Jet printer (BIJ-600) equipped with a standard ink cartridge [28]. The optimal “ink” composition was evaluated regarding volatility, solubility, wettability, viscosity, and surface tension, and empirically an aqueous solution of glycerol, urea, thiodiglycol (these three

serve as wetting agents), and acetylenol (reduction of viscosity) was chosen as the solvent for DNA printing. The use of ethanol (approx. 10% v/v) as an additive to reduce viscosity and surface tension of inks containing biological samples, which improves the printing reliability by facilitating the wetting of the internal capillary of the ink cartridge, is also possible [34]. Particularly, the viscosity of the ink is a crucial parameter in handling fragile biological samples, because it determines the shear stress experienced by the ink during the droplet ejection process. Although exposure to heat (200–300 °C) and shear rate (10^4 s^{-1}) were a concern in terms of possible DNA degradation, ejected DNAs of 10–300 base pairs (bp) in length and 0.02–1.6 mg mL⁻¹ in concentration remained intact. Quite the contrary, the authors found an advantage of heating the DNA-containing ink. They assumed that the high temperature and the presence of urea in the ink induced DNA denaturation, providing the reaction energy required for a high rate of DNA binding to the glass substrate surface (completed within 30 min). The robustness of DNA compared to other biological substances such as proteins is attributed to its 3D structure. Hydrogen bonds formed between base pairs of complementary DNA strands determine its architecture univocally without bending or folding. On the other hand, proteins are much more challenging to inkjet print without ruining their higher-order structures (see the following section for details about inkjet printing of proteins).

The power of inkjet printing of nucleic acids is particularly revealed in inkjet-based solid-phase synthesis of DNA, which is another powerful technique to align DNAs on a solid support. It involves *in situ* DNA synthesis on a print medium in a manner similar to the photolithographic approach by delivering DNA nucleotides (monomers). In this approach, DNA nucleotides (typically phosphoramidite monomers) of the four bases are selectively and precisely deposited only onto the desired spots from independent nozzles. Typical inkjet-based *in situ* DNA synthesis using phosphoramidite protection chemistry is schematically depicted in Fig. 8.5. This method eliminates the requirement of photomasks for spatial control and enables designing of more than tens of thousands of sequences with a single inkjet printer by simply modifying the printing pattern using computer software. Despite the disadvantage that the obtainable length of DNA is shorter than in the case of the abovementioned pre-synthesized DNA printing, this approach possesses significant merits over the complete DNA printing method: (1) theoretically, any sequence can be obtained by one printer without replacing ink cartridges, (2) DNA sequences can be stored as electronic data files instead of frozen DNA libraries. Although several routes of solid phase DNA synthesis have already been reported in the 1980s [35–37], transfer to inkjet printing was first demonstrated in 1996. A research group at the University of Washington attempted the delivery of DNA monomers onto a silicon dioxide wafer patterned by using a self-made piezoelectrically actuated inkjet pump. Though the performance of the DNA array itself has not been specified, the throughput achieved with this approach allowed for the arraying of 100,000 arbitrary 25-mers within only 2 h [38]. A next step in the evolution of this technology was triggered in 2001 by research groups at Rosetta Inpharmatics and Agilent Technologies, two primary inkjet-printed DNA array manufacturers. Following the first *in situ* DNA synthesis on a glass support employing a self-made inkjet device,

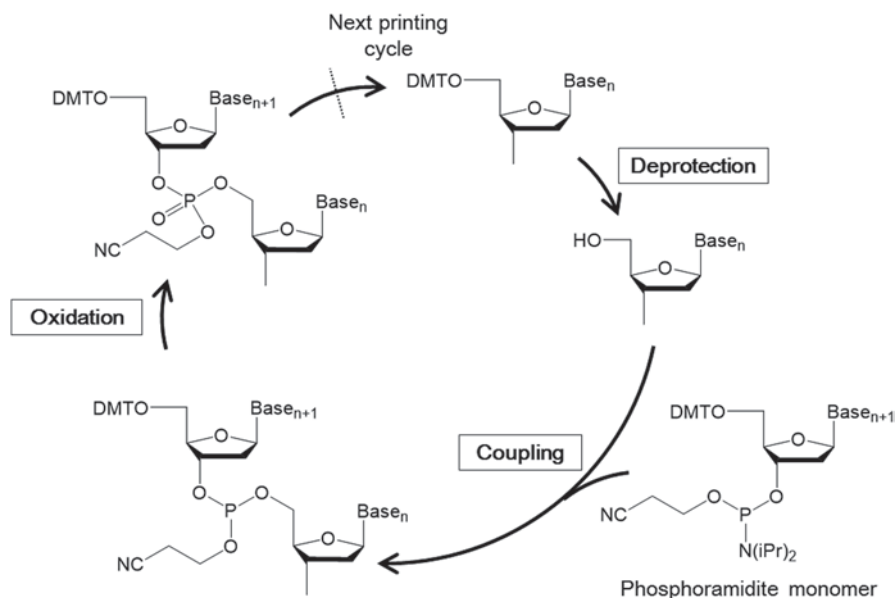


Fig. 8.5 Typical in situ synthesis cycle employing phosphoramidite chemistry. *DMT* dimethoxytrityl protection for the hydroxyl group

this method was further optimized and validated. The combination of standard phosphoramidite DNA synthesis and commercially supplied inkjet printer heads (product name not specified) enabled the creation of 25,000 different 60-mers on a $25 \times 75 \text{ mm}^2$ glass support with stepwise synthetic yields (one cycle in the process depicted in Fig. 8.5) of 94–98% [29]. This amount of simultaneously arrayed oligonucleotides covers the estimated number of 20,000–25,000 human genes [39]. At present, the stepwise synthetic yield has been improved to values as high as 99.5%, and longer DNAs became obtainable with smaller failure rates (successful synthesis rate has risen from 20 to 60% in the case of 100-mers) [40]. The versatility of the present method is accelerated by the introduction of an open-source inkjet arrayer in 2004. In spite of the strong potential of inkjet-based in situ DNA synthesis, information on this technology accessible to the general research community (not inkjet-related enterprises) was relatively limited compared to other arraying technologies. As for the pin printing method, the arrayer design was released earlier in 1998, which fell in the middle of the Human Genome Project. Thus, it became widely and readily available and triggered multiple research efforts and product developments in the field of microarrays. In this context, The Institute for Systems Biology came up with the Piezoelectric Oligonucleotide Synthesizer and Microarrayer (POSaM), a piezoelectric inkjet dispenser assembled from mostly off-the-shelf components except for circuit boards and some other minor components [41]. This system is capable of printing 9800 different oligonucleotides on an 8 cm^2 area of modified glass and of handling up to 27 slides in parallel. The printhead used is fed by six ink

channels (four phosphoramidite monomers, ethylthiotetrazole as an activator, and optional linker or modified base), and dispenses droplet volumes as small as 6 pL with 100 μm in dropped feature size and 280 μm in dropping space. In addition to the construction of an easily accessible inkjet arrayer, the same work describes some important efforts to fabricate high quality microarrays. First, suitable ink solvents were newly investigated, and a 1:1 mixture of methyl glutaronitrile (MGN) and 3-methoxypropionitrile (3MP) was found to be optimal in terms of volatility, solubility, and surface tension. Acetonitrile is commonly used in automated phosphoramidite synthesis, but its high volatility is disadvantageous to provide proper reaction sites for coupling and results in the reduction of synthetic yield. Furthermore, it can cause clogging of the print head by forming phosphoramidite precipitates during long period storage. Hence, propylene carbonate was chosen as an alternative solvent to accommodate in situ oligonucleotide synthesis by inkjet printing, but a relatively low synthetic yield of 94–98% for each step significantly affects the array quality in this serial procedure. By dissolving the nucleotides in the 1:1 mixture of MGN and 3MP, the synthesis efficiency was increased to $97.1 \pm 1.2\%$, which is comparable to acetonitrile ($97.2 \pm 1.5\%$) and superior to propylene carbonate ($93.0 \pm 1.3\%$) [41]. Second, a laser diode droplet detector was incorporated into the system as an effective measure to monitor nozzle ejection failures. Successful droplet ejection is confirmed based on light beam scattering by droplets, detected by digitally converted photodiode signals (Fig. 8.6). Failing nozzles are omitted from use depending on the ejection status checked at the beginning of each printing cycle, and the software recalculates the most efficient printing path, accordingly. Detection and elimination of defective ejection is critical in preparing arrays reproducibly, since otherwise the entire array might be compromised. In addition to this function, the system allows for environmental control (humidity, inert gas supply) and precise motion control features. Despite the high setup costs (\$ 34,000), the POSaM system has various advantageous characteristics for flexibly designing high quality inkjet-printed DNA arrays.

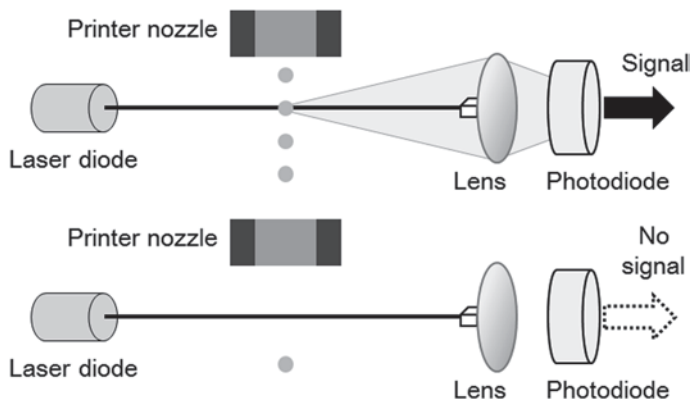


Fig. 8.6 Droplet monitoring system in a POSaM: **a** normal ejection, **b** defective ejection. (Adapted from reference [41] (<http://genomebiology.com/2004/5/8/R58>); © 2004 Lausted et al.; licensee BioMed Central Ltd)

8.3.4 Inkjet Deposition of Nucleic Acids for Miniaturized Devices

In the future, inkjet-based deposition of nucleic acids will probably play an increasingly active part in developing novel miniaturized analytical devices, rather than in the already technically mature preparation of DNA microarrays.

One early example of applying inkjet printing of DNA for such objective is the fabrication of cantilever sensors. An IBM research group has reported on the functionalization of gold-coated cantilevers with thiol-linked 12-mer oligonucleotides for sequence-specific detection of DNAs using a piezoelectric inkjet dispenser MD-P-705-L (Microdrop, Norderstedt, Germany) [42]. A total of 100–300 nL droplets (corresponding to diameters of 60–80 μm in air) of three different 12-mer oligonucleotides were spotted onto a cantilever array (250 μm pitch), as small as 500 μm in length and 100 μm in width without cross-contamination between adjacent cantilevers.

Yasui et al. applied a piezoelectric inkjet system to inject DNA samples into a microchip device for electrophoresis [43]. With the objective of avoiding denaturation of DNA, they employed a piezo-based injector (Pulse Injector from Cluster Technology Co., Ltd.), and succeeded in handling DNAs up to 3000 bp at a concentration of 4.9 ng/mL. Generally, cleavage is much easier to occur for long DNA molecules. Indeed, in a cleavage study by gel electrophoresis, they confirmed a tailed band in the case of λ DNA (48,502 bp) after the injection process, while 250 and 1000 bp DNAs showed bands identical to those of a control sample (not subjected to the inkjet process).

Although the factors determining the maximum printable oligonucleotide length are not fully investigated (e.g., influence of DNA concentration, stress experienced in the droplet ejection process, etc.), the possibility to precisely and reproducibly handle small volumes of relatively long nucleic acid solutions is definitely helpful in developing miniaturized analytical platforms such as microfluidic or nanofluidic devices. Such miniaturized analysis systems are now of great interest especially in medical diagnostic fields, because they are fast, enable the reduction of analytical sample volumes, and material costs for fabrication.

8.3.5 Substrates for Nucleic Acid Immobilization

Immobilization of nucleic acids has been attempted for several purposes such as the manufacturing of microarrays and the development of sensors. Some examples of substrates used for immobilizing nucleic acids are listed in Table 8.3. Owing to their widespread availability, robustness, ease of derivatization, and low intrinsic fluorescence, glass microscope slides have been widely used and represent the most common platform for microarrays. In some cases, more sophisticated platforms like quartz glass, silicon wafers, and gold-coated surfaces are applied. These surfaces are normally pretreated to bear functional moieties for coupling with DNAs or their monomers by well-established chemistries (e.g., amine–epoxy, gold–thiol, avidin–biotin linkages) described in detail elsewhere [21, 44]. Figure 8.7 shows an example

Table 8.3 Substrates for inkjet printing of nucleic acids

Substrate	Substrate modification	Immobilization principle of DNA
Microscopic glass slide [30, 41, 45]	Hydroxylation ^a [30] Epoxydation [41] Aminization ^a [45]	Covalent linkage
Quartz glass [28]	1) Amination 2) Maleimidation	Covalent linkage (maleimide-thiol DNA)
Silicon wafer [29, 38]	Hydroxylation ^b	Covalent linkage (hydroxyl group-phosphoramidite)
Nitrocellulose [47]	Baking ^c at 80 °C	Hydrophobic interaction
Nylon membrane [47, 48]	Baking ^c at 80 °C Blocking ^c treatment	Electrostatic adsorption
Zetaprobe membrane ^d [48]	Blocking ^c treatment	Electrostatic adsorption
Gold thin film [42]	Au coating of substrate	Gold-thiol linkage
Cyclic olefin copolymer [51]	Silicon oxide sputtering	Covalent linkage (hydroxyl group-phosphoramidite)

^a Fluorination was performed to prepare discrete spots by utilizing the difference of surface tensions

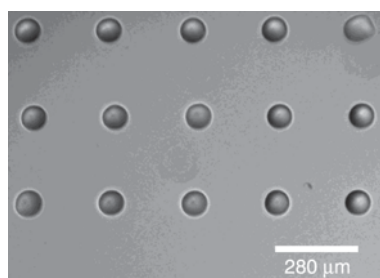
^b Perfluorination was performed for the same purpose as described above

^c Run after DNA print

^d Nylon-based membrane with high mechanical strength and positive charge derived from quaternary amine groups (provided by Bio-Rad)

of droplets of phosphoramidite nucleotides in 1:1 3MP:MGN POSaM printed onto a standard epoxysilane-modified glass slide surface [41]. The hydrophobic nature of the silanized glass surface and the hydrophilic ink droplets results in the formation of “virtual reaction wells” for in situ DNA synthesis. To improve the homogeneity of inkjet-printed DNAs, the patterning of round-shaped areas occasionally called “surface tension wells” on the print support has been proposed [38, 45, 46]. The surface tension well is surrounded by highly hydrophobic zones (typically a fluorinated surface obtained by photolithography) and acts as a discrete hydrophilic coupling reaction site. This feature contributes to the improvement of print spot quality, depending on the surface tension of the printing ink or the precision of the inkjet printer. Lausted and co-workers compared spot alignment and spot morphology printed by a POSaM onto two kinds of substrates: (1) untreated glass slides and

Fig. 8.7 Virtual reaction wells on silanized glass (phosphoramidites and tetrazole dissolved in 1:1 3MP:MGN), (From reference [41]. (<http://genomebiology.com/2004/5/8/R58>); © 2004 Lausted et al.; licensee BioMed Central Ltd)



(2) hydrophobic silicon slides with predefined hydrophilic wells (Lumera-patterned silicon). In the latter substrate, hydroxyl group-rich regions are circularly surrounded by a polydimethylsiloxane (PDMS) surface. Spots on this substrate are perfectly round shaped with uniform diameters. On the other hand, spots on the untreated glass slide show somewhat inhomogeneous shapes, and incorrect positioning.

Porous materials have also been studied as solid supports for inkjet printing of nucleic acids. For instance, nitrocellulose and nylon membranes are known as common membrane supports for the blotting of nucleic acids and proteins. They possess a fibrous structure with micropores of 0.45 μm in diameter and negatively or positively charged surfaces derived from nitrate groups and quaternary ammonium groups, respectively. Nucleic acids with their negatively charged phosphate groups strongly adsorb onto nylon membranes electrostatically. Some groups attempted transferring DNA samples onto these supports by using commercial personal desktop inkjet printers. DNA solution was filled into emptied and cleaned ink cartridges and printed on membranes fed into a thermal inkjet printer. Labeled cDNAs [47] and PCR-amplified DNA [34] were transferred onto nitrocellulose, nylon membrane, and Zetaprobe membrane (high-strength cationized nylon membrane with high-density quaternary amine charge; commercial product of Bio-Rad), respectively and sequence-specific hybridization signals were successfully observed. Furthermore, it has been indicated that Zetaprobe membranes showed less nonspecific background binding and higher signal in comparison to common nylon membranes in a fluorescence-based hybridization test [48]. These membranes are commercially available and compatible with common vertical loading inkjet printers, thus are easily accessible alternatives for the inkjet-based fabrication of DNA hybridization assays. However, employing fibrous materials as arraying substrates is challenging mainly due to two reasons: first, it is difficult to obtain high-density arrays because droplets after impact on the substrate are likely to spread automatically via capillary forces. This can be avoided by pre patterning porous materials in a similar manner to “surface tension wells.” Various approaches for hydrophobic patterning of porous materials (filter paper, chromatography paper, etc.) have been developed so far [49], but almost all of them are not resistant to organic solvents, hence considerably limiting the available DNA “ink” composition. Second, in situ DNA synthesis yields achievable in fibrous networks might be quite low due to limited contact between reactants. This means that quantitative DNA arrays with various sequences are virtually unrealizable on such substrates.

Only comparatively recently, synthetic polymeric materials have been successfully applied as a substrate for in situ DNA synthesis by inkjet printing. Interest in polymeric platforms is particularly driven by the fact that the traditional glass or silicon substrates are not easily adaptable for the integration into microfluidic systems [50]. Saaem et al. employed cyclic olefin copolymer (COC) as inexpensive, yet physically, chemically, and optically preferable substrate for DNA microarrays. They succeeded in preparing spot arrays onto an originally highly hydrophobic and chemically inert COC surface by sputtering a silicon dioxide thin film onto microwells created by photolithography (Fig. 8.8). Then, DNA synthesis was performed in each well by delivering phosphoramidite nucleotide monomers using a POSaM.

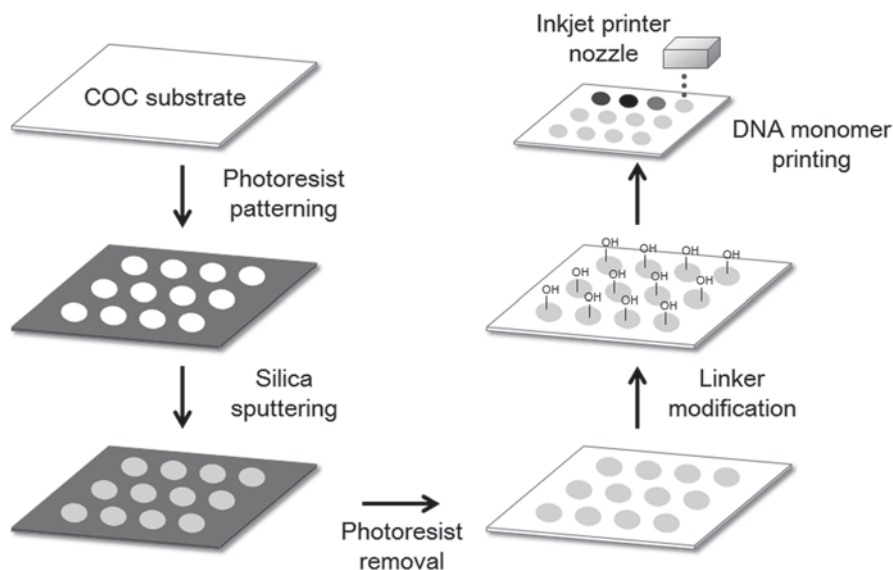


Fig. 8.8 Procedure of in situ DNA synthesis on polymer substrate by inkjet printing

Resulting array features and oligonucleotide density were comparable to typical glass (50 μm spot diameter in 100 μm pitch and approximately 0.8–1.2 molecules per μm^2 [51]). It has to be noted, however, that this approach required the local chemical conversion of the substrate surface from its original synthetic polymeric state into a glasslike structure. To the best of our knowledge, an inkjet printing-based approach to in situ DNA synthesis on an unmodified synthetic polymeric platform has yet to be demonstrated.

8.4 Inkjet Printing of Proteins

8.4.1 Introduction

Development of a printing strategy that could deposit multiple different proteins, such as enzymes and antibodies, as biorecognition elements on a common polymeric surface without loss of activity and structural integrity is essential in the advancements of a plethora of applications such as drug screening, clinical diagnostics, environmental monitoring, food analysis, biomarker discovery, tissue engineering, and proteomics research [16, 52, 53]. Proteins are biomacromolecules with primary structure composed of a repertoire of amino acids linked together by peptide bonds. This linearly chained protein structure assembles to form the secondary structure which is either helical, globular, or sheet folded through intramolecular hydrogen bonding. Then, the interaction of the secondary structures could mold the functional tertiary structure of the protein [54]. In contrast to the much more stable structures

of nucleic acids, the fragility of the protein structure is not trivial in the area of protein immobilization. Even a slight change in the 3D protein structure could have a significant effect on its function. Usually proteins are operated at their optimum condition to preserve the 3D structure and conformation. Some of the factors that could affect the protein structure in an aqueous solution are temperature, pressure, pH, ionic strength, and organic modifier. In case of protein immobilization, the surface property of the solid material could also induce a change in the protein structure. Therefore, proper handling is critical to achieve an active printed protein on any solid substrate, including polymeric platforms.

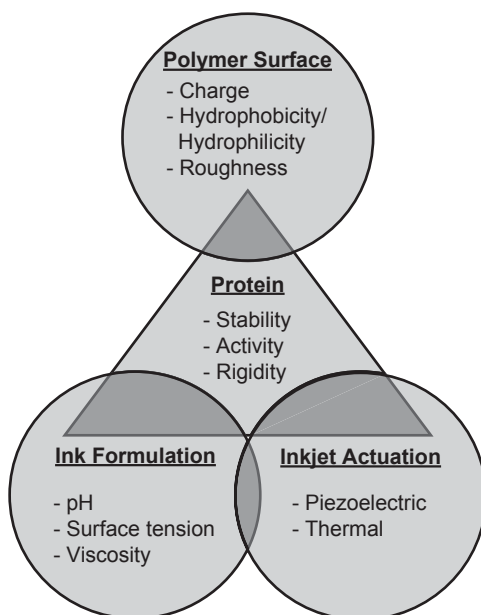
The noncontact characteristic of inkjet printing technology makes it potentially a material deposition technique that could gently deliver delicate proteins on a material surface. The mechanism of ink jetting has been discussed thoroughly further above. Essentially, the protein ink solution in the printer cartridge experiences a microsecond pressure pulse and is ejected through the orifice of the printhead nozzle onto the printing substrate located at a distance of 1–5 mm. In terms of versatile protein printing on different polymeric platforms, the inkjet approach is highly suitable for biomolecule deposition due to the following reasons: (1) the contactless operation could significantly minimize protein denaturation; (2) the miniscule picoliter droplet ejection may lead to minimal consumption of precious protein reagent; (3) the highly position-specific printing could lead to precise and reproducible protein immobilization; and (4) the multi-cartridge system of commercial office desktop inkjet printers could potentially lead to high-throughput multiplexing and cost-effective, mass producible protein immobilization.

In this section, focus is directed towards the inkjet deposition of enzymes or antibodies on various substrates with emphasis on polymeric platforms. This also includes some discussion on surface modification and mechanisms of protein immobilization to achieve a surface for successful selective biorecognition. A brief description on the nature of enzymes or antibodies is given to understand the importance of the protein immobilization. Figure 8.9 shows a schematic general overview of this section. Basically, the stability, sensitivity, and structural integrity of protein molecules are dependent on three basic factors—printer actuation, ink formulation, and nature of the substrate surface. Research work devoted to investigating factors that affect the protein stability and activity will also be discussed. Eventually, an outlook on the future direction of this field will be presented.

8.4.2 Inkjet Printing of Enzymes

Enzymes are protein molecules that function based on the activity of the catalytic site and retention of its conformation. In general, the enzyme structure is a combination of hydrophobic amino acid (e.g., phenylalanine, tyrosine) core region and basic (e.g., lysine, arginine) or acidic (e.g., aspartic acid and glutamic acid) hydrophilic amino acid outer surface structure [55]. As a consequence, enzymes are amphiphilic biomaterials that could act both as hydrophobe and hydrophile with a net surface charge depending on the solution pH and isoelectric point. Various types of

Fig. 8.9 Overview of the factors affecting the inkjet printing of proteins



non-covalent interactions are involved to preserve the active form of the enzyme, such as hydrogen bonding, van der Waals forces, salt bridges, and hydrophobic interactions. Moreover, the rigidity of an enzyme, which is dependent on the protein structural flexibility, has also an important role on its stability. Hard enzymes have a rigid structure that could maintain the conformation upon adsorption to a surface. On the contrary, significant loss in activity could be observed for a soft enzyme due to disruption of the non-covalent interactions [56]. These characteristics of enzymes are important to have a well-thought approach on how to print them on a polymeric platform.

In 1988, Kimura et al. have successfully demonstrated the first inkjet printing of multiple enzymes, such as glucose oxidase (GOx) and urease, on a silicon wafer surface by creating a glutaraldehyde-crosslinked enzyme membrane for ion-sensitive field effect transistor (ISFET) application [57]. The native silicon wafer surface was used without further treatment. They have incorporated bovine serum albumin (BSA) in the ink formulation to minimize the denaturation of the GOx or urease. Also, the glutaraldehyde molecule may have increased the rigidity of the enzyme structure that led to maintaining its activity and at the same time created an entrapped enzyme within the predominantly BSA-crosslinked membrane. Figure 8.10a shows the experimental setup of the first inkjet printer with a single cartridge used for enzyme printing. Both the X-Y stage and pulse generator were controlled by the computer. It is important to note that the protein droplet-impact position was guided by the microscope and not initiated by the computer software. A photograph of the actual pattern of protein membranes on an ISFET device is depicted in Fig. 8.10b. By confirming an electrochemical response of the device

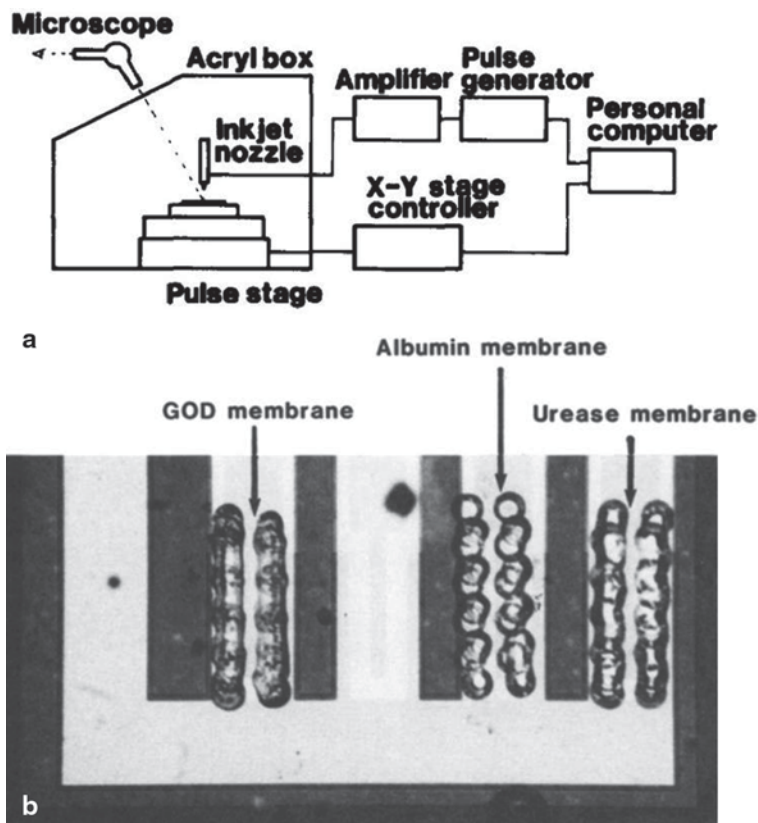


Fig. 8.10 First demonstration of protein printing by inkjet technology: **a** inkjet printer setup; **b** protein membranes deposited on an ISFET device. Reprinted from Kimura et al. (1988) *Biosensors* 4:41–52, Copyright (1988), with permission from Elsevier [57]

to glucose and urea, it was indirectly proven that such enzyme deposition strategy could preserve the activity of GOx and urease. Indeed, the inkjet printing technology was in its infancy during that time and its potential for high-resolution printing and mass production has not been realized.

By 2000, inkjet printing technology has matured significantly and commercially available office desktop printers have been exploited for enzyme immobilization. Roda et al. were among the first to demonstrate such use of an office desktop inkjet printer by depositing horseradish peroxidase (HRP) on various solid supports such as conventional cellulose paper with different weights (30–80 g/m²), cellulose filter paper, nylon sheet, photographic gelatin paper, tissue paper, and inkjet transparency film [58]. The printed HRP was simply adsorbed on the surface of the polymeric material and evaluated by chemiluminescence (CL) detection. Among the polymeric platforms, it was the lightest conventional cellulose paper (30 g/m²) that resulted in the maximum CL intensity with the shortest diffusion time of the CL substrate. Figure 8.11 shows the effect of the spot diameter and activity of the HRP enzyme

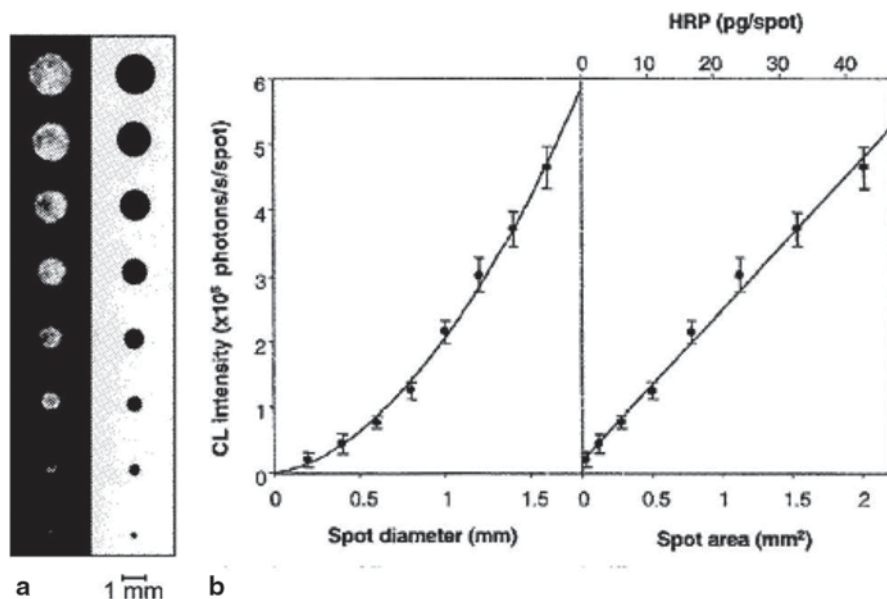


Fig. 8.11 **a** Left part shows the chemiluminescence signal and right part shows the deposition pattern of an array of HRP spots with different diameters on conventional cellulose paper. **b** Correlation of the chemiluminescence signal and spot diameter, spot area, and amount of HRP. Reprinted from reference [58] Roda et al. (2000), Protein microdeposition using a conventional inkjet printer. *Biotechniques* 28:492–496 © 2000 BioTechniques. Used by Permission

deposited on the cellulose paper surface. Based on this early work, the feasibility of enzyme deposition using conventional inkjet printing on various material surfaces was realized. The delay in the advancement of protein printing by inkjet technology was probably due to its digital printing nature which relies on the development of both the software and hardware to achieve an efficient transfer of computer graphics onto the printing substrate.

Most of the current investigations on enzyme stability during inkjet printing are focusing on actuation-related parameters (e.g., ink compression [59], shear rate [60], and thermal effect [61]), and ink formulation (e.g., viscosity modifier [62]). Nishioka et al. have studied the effect of inkjet printing on the HRP enzyme by exposing the protein to various compression rates (85 V applied on a piezoceramic between 14 and 70 μ s) generated by piezo-actuation [59]. They have demonstrated that compression of the ink liquid could have a direct effect on the HRP activity. Commercial desktop inkjet printers have a drop velocity of about 10 m/s, which creates a condition of high compression rate, thus possibly affecting the stability of the enzyme. However, the use of sugars like trehalose/glucose as ink additives could act as cushion, through extensive hydrogen bonding, to protect the protein from such pressure pulse. In a different report, it was shown that the shear effect of piezoelectric inkjet printing on GOx has no significant effect on the secondary and tertiary structures of the protein, but degradation in enzyme activity was observed

[60]. The authors hypothesized that low level of structural distortion could have affected the active site and the enzyme–substrate interaction. In case of thermal inkjet printing of enzymes, it seems natural to assume that loss in enzyme activity may occur due to high temperature (200–300 °C) exposure in the printhead's firing chamber during vapor bubble formation. Interestingly, based on the work of Khan et al., such effect was insignificant at least when printing an HRP enzyme [61]. This could be due to the following reasons: (a) as already mentioned above, the exposure time of the ink to high temperature is only in the order of a few microseconds and (b) assuming the distance of the printhead and substrate is about 2 mm and the drop velocity is 10 m/s, it takes about 200 μ s for the droplet to reach the substrate surface being at room temperature. The remaining heat confined in the droplet could be absorbed by the paper cellulose which has a high heat capacity [2]. Therefore, the overall exposure time to high temperature is probably too short to induce damage on the enzyme activity. On the other hand, the influence of ink viscosity modifiers has been investigated by exposing the HRP enzyme to a number of polymers with different molecular weights, and functional and ionizable groups [62]. Using a piezoelectric inkjet printer for HRP printing, it was concluded that carboxymethyl cellulose, among other polymers, was found to maintain the enzyme activity at a viscosity similar to commercially available inkjet inks (2–9 cP). Here, it is important to keep in mind that the balance between jetting parameters and ink formulation should always be considered when printing an enzyme. This has been pointed out by Arrabito et al. [63], who demonstrated that an increasing amount of glycerol in the ink may support a uniform protein pattern and enzyme stability, but at the same time may also result in higher shear rates on the protein that may eventually affect the enzyme activity.

Table 8.4 shows some of the current reports on enzyme deposition by inkjet printing on various substrate material surfaces. Thermal- and piezoelectric-actuated inkjet printers are both used for enzyme immobilization. Apparently, piezoelectric actuation is utilized more often than the thermally actuated jetting. Although there is a report on insignificant loss in enzyme activity using thermal inkjet printing [61], the piezoelectric approach is probably assumed to offer a more gentle way of actuation, since the bio-ink solution is not exposed to extreme heat. However, it is important to remember that piezoelectric actuation requires higher ink viscosity (5–10 cP) than the thermal inkjet printing (1–1.5 cP) (Table 8.1), which means that an enzyme in ink formulations for piezo-based printers could experience higher shear stress during jetting. Both piezoelectric and thermal actuation have their own advantages and disadvantages, and they always require an optimization of their parameters to achieve a successful enzyme deposition. Moreover, a number of enzymes and polymeric materials have been used for inkjet printing (Table 8.4). The generally used polymeric substrates are cellulosic filter papers, glass, and plastics (e.g., polystyrene, polypropylene, and polyethylene terephthalate (PET)). These are low-cost and readily available materials. Adsorption is perhaps the most convenient method of enzyme immobilization to the majority of these surfaces. This is mainly because of its simplicity by directly printing on the polymer surface without any chemical modification or treatment. Since proteins are amphiphilic biomaterials, their surface

Table 8.4 Enzyme immobilization on various materials by inkjet printing

Substrate material	Enzyme	Inkjet actuation	Base ink for enzyme	Enzyme immobilization	References
Si wafer	Glucose oxidase (GOx), Urease	Piezoelectric	10 mM HEPES buffer (pH 7.5), 3–5% bovine serum albumin (BSA)	Entrapment	[57]
Filter paper or plastic	Horse radish peroxidase (HRP)	Thermal	0.1 M Tris-HCl buffer (pH 8.6), 1.5 mM sodium dodecyl sulfate (SDS), 10 mM NaCl	Adsorption	[58]
Plastic	Peroxidase	Piezoelectric	Buffer, trehalose/glucose	Adsorption	[59]
Glass	GOx	Thermal	0.1 M Phosphate buffer (pH 6.5), 1.5 mM EDTA, 10% glycerol	Adsorption	[67]
Glass	GOx	Piezoelectric	Vinylacetate/ethylene copolymer	Adsorption	[68]
Glass	HRP	Thermal	0.1 M Phosphate buffer (pH 6.5), 1.5 mM EDTA, 10% glycerol	Adsorption	[69]
Polyvinylidene difluoride	Trypsin, PNGase F	Piezoelectric	25 mM Ammonium bicarbonate, 10% 2-propanol	Adsorption	[70]
Silicon dioxide	GOx	Piezoelectric	0.05 M Phosphate buffer (pH 6.5), 0–50% glycerol	Covalent	[63]
Filter paper	ALP/HRP	Thermal	0.1 M Phosphate buffer (pH 6.0)/1 M diethanolamine buffer, 0.5 MgCl ₂	Adsorption	[61]
Filter paper	AChE	Piezoelectric	100 mM Tris buffer (pH 8)	Entrapment	[65]
Plastic	GOx, glutamate and glucose dehydrogenase	Piezoelectric	Phosphate buffered saline (PBS)	Adsorption	[60]
Filter paper, electrospun poly-(ε-caprolactone)	HRP	Thermal/ Piezoelectric	0.1 M Phosphate buffer (pH 6)	Adsorption	[64]
Screen-printed carbon paste	Urease	Piezoelectric	0.1 M Phosphate buffer (pH 7.12), 0.1% glycerol, 0.01% Triton X-100	Adsorption	[71]
Polyethylene terephthalate (PET)	GOx, HRP	Piezoelectric	PEDOT/PSS in water	Adsorption	[72]

Table 8.4 (continued)

Substrate material	Enzyme	Inkjet actuation	Base ink for enzyme	Enzyme immobilization	References
Filter paper	Tyrosinase	Piezoelectric	0.1 M Phosphate buffer (pH 6.5)	Entrapment	[66]
Silicon dioxide	CY3A4	Piezoelectric	0.1 M Phosphate buffer, 30% glycerol	Adsorption	[73]
Filter paper	AChE	Piezoelectric	Tris buffer, 30% glycerol, 0.1% Triton X-100	Entrapment	[74]
PET	GOx	Piezoelectric	20 mM Acetate buffer (pH 5.5)	Adsorption	[75]
Screen-printed carbon electrode	GOx or HRP	Piezoelectric	Polypyrrole in water	Adsorption	[76]

AChE acetylcholine esterase

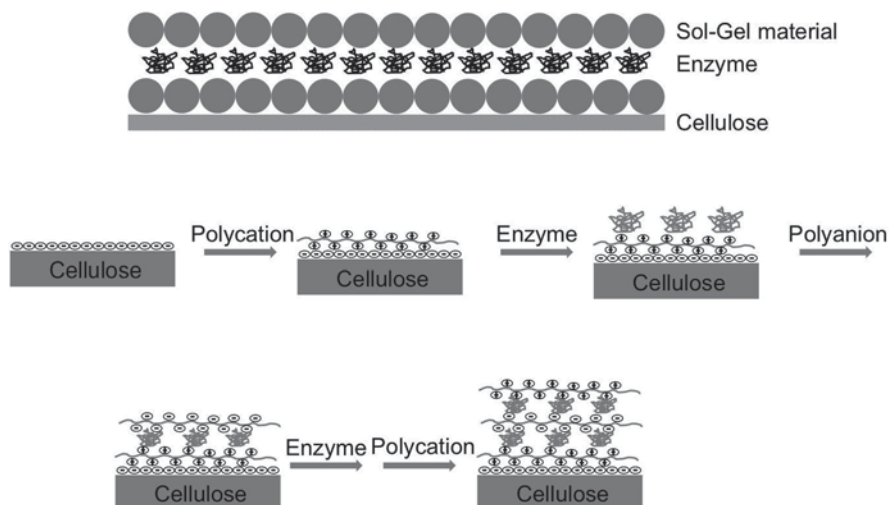


Fig. 8.12 Enzyme entrapment by **a** sol–gel material (adapted with permission from Hossain SMZ et al. (2009) *Anal Chem* 81:5474–5483. Copyright (2009) American Chemical Society [65]). **b** Layer-by-layer method (adapted with permission from Alkassir et al. (2012) *Anal Chem* 84:9729–9737. Copyright (2012) American Chemical Society [66])

adhesion is governed by either hydrophobic (van der Waals forces) or hydrophilic (hydrogen bonding/electrostatic) interactions or their combination. However, one of the important requirements to achieve an optimal surface wetting and efficient adhesion of the protein is to have an ink with lower surface tension than the surface energy of the substrate material [64]. A surfactant such as Triton X-100, a nonionic molecule and mild detergent, is an example of a reagent that could lower the surface tension of the ink formulation. At least when included in an ink formulation containing acetylcholine esterase (AChE), 0.1 wt% Triton X-100 had no effect on the activity of printed AChE [65]. Furthermore, an approach to increase the surface energy of a substrate for printing is a surface pretreatment or modification. Sequential printing of reagents involving sol–gel applications or layer-by-layer (LbL) deposition are some of the interesting approaches that have been explored to modify the surface property of the substrate for a more stable protein immobilization [65, 66]. For sol–gel printing, the silica sol was printed separately from the enzyme solution (Fig. 8.12a) to avoid the gelation to occur in the nozzle of the inkjet printer. It was confirmed that the sol–gel-based ink was deposited on the surface and that enzyme entrapment was effective. In a different approach, LbL deposition of chitosan and sodium alginate with tyrosinase enzyme sandwiched in between layers was accomplished by inkjet printing (Fig. 8.12b). Initially, pentabasic sodium triphosphate (NaTPP) was adsorbed on the filter paper to act as a cross-linker and stabilizer of cationic chitosan. This type of enzyme immobilization provided a biocompatible microenvironment and electrostatic anchoring for the enzyme, which led to a stable bioactive structure on the filter paper surface. Given the proper rheological properties of the bio-ink, both sol–gel enzyme deposition and LbL approach could provide simple fabrication, enzyme-friendly microenvironment, retention of activity, and potential for mass production.

8.4.3 Inkjet Printing of Antibodies

Antibodies, also known as immunoglobulins, are large glycoprotein molecules that have high specificity for their antigens which could be a protein, polysaccharide, or lipid. Their basic structure is almost Y-shaped, with two arms composed of pairs of heavy and light polypeptide chains and a trunk made up of a pair of heavy polypeptide chains [77, 78]. The pairs of polypeptide chains are covalently connected by disulfide bonds. This covalent bond controls the structural rigidity and flexibility of the antibody. Antibody–antigen interactions are an extremely important form of biorecognition and are used in various applications such as medical, clinical, pharmaceutical, and proteomics. However, most antibodies are expensive and available in extremely low amounts. A microarray format is the commonly used type of analytical device to maximize the use of miniscule amounts of antibodies on a given substrate surface. Therefore, inkjet printing can offer a convenient and practical solution to the task of dropping a picoliter amount of expensive antibodies in a small, defined region of a substrate surface.

In 1995, Nilsson et al. reported the concept of thin-layer immunoaffinity chromatography, which was an early demonstration of piezoelectric antibody inkjet printing onto a pre-activated nylon membrane for the analysis of the disease biomarker C-reactive protein (CRP) [79]. A monoclonal antibody (Mab) in three different concentrations was printed in a line configuration and formed three selective biorecognition zones (Fig. 8.13). The immunocomplex solution of CRP antigen-Mab-coated latex traveled across the membrane, and then formed blue-colored lines due to sandwich immunocomplexes formed with the printed antibody in the biorecognition zones. This result proved the feasibility of inkjet printing of antibodies on a

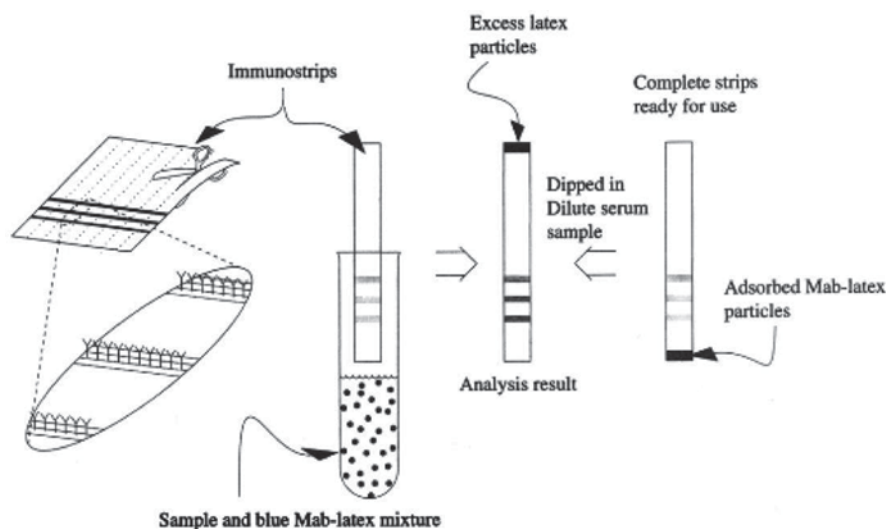


Fig. 8.13 Line pattern deposition of antibodies on a nylon membrane by a customized inkjet printer. Reprinted with permission from Nilsson et al. (1995) *Anal Chem* 67:3051–3056. Copyright (1995) American Chemical Society [79]

polymeric support. However, it was only around 2008 that research publications about antibody inkjet printing revived the interest in the topic among the academic community. Historically, the antibody microarray technology is an offshoot of the DNA microarray technology, which was already mature in the early 1990s. This DNA microarray technology was directly translated to fabricate antibody microarrays. Therefore, there was not much research output and efforts only focused on the routine implementation of the commercially available microarrays. Recently, there are demands for the development of antibody microarrays to be more cost-effective and easy to fabricate while maintaining throughput and minimal reagent consumption. Commercially available office desktop inkjet printers could be a potential solution to this challenge. Lonini et al. have used both HP Deskjet 5740 (thermal) and EPSON Stylus c46 (piezoelectric) to investigate the effect of these office printers on immunoglobulin dispensing for an immunoassay [80]. They compared both manual and inkjet printer-based deposition of antibodies on a plastic microwell plate. The use of the piezoelectric printer resulted in a more convenient operation, and comparable immunoassay results with the manually dispensed antibodies were achieved. Meanwhile, the thermally actuated printer did not allow for a reproducible deposition of the antibodies. This result suggests that the piezoelectric actuation is preferred over the thermal actuation.

Table 8.5 describes some of the recent developments in antibody microarray fabrication by using piezoelectric inkjet printers. Apparently, piezo-actuation is the favored mode of fluid dispensing for antibodies, as briefly discussed above. Besides the ink formulation and printer actuation, the surface property of the polymer substrate is also an important factor to consider when printing an antibody. Depending on the surface, the antibody could interact through adsorption, covalent binding, molecular recognition, or entrapment in a porous material (Fig. 8.14). Representative reports are shown in Table 8.5. Molecular recognition requires an initial deposition of a biorecognition element such as avidin or streptavidin to be able to immobilize the biotinylated antibodies. This manner of immobilization allows efficient orientation of antibodies to easily form an immunocomplex. Unlike enzyme printing, which utilizes adsorption as the main method of immobilization, the recent trend in antibody immobilization is through covalent bonding. This is probably due to the nature of the related application. Printed antibodies are generally used in biosensors that require multiple bioassay steps such as immunoreaction and washing. This operation necessitates fluid handling with flowing liquids; thus, it may be important that antibodies are permanently attached on the substrate material. Although it is common practice to allow antibodies to be adsorbed on substrates like nitrocellulose, it has been observed that covalent immobilization allows a more wash-stable protein pattern, since fluid movement might promote desorption of molecules from the surface. Moreover, covalent immobilization offers an opportunity to control the proper orientation of antibodies for the immunoreaction.

Polymer substrates used for antibody microarray fabrication can be divided into two types: porous and nonporous materials. Glass and polystyrene are the most generally accepted nonporous substrates, since they are easily available and inexpensive. Mujawar et al. have investigated the effect of changing the surface

Table 8.5 Antibody immobilization by inkjet printing on various materials

Substrate material	Antigen	Inkjet actuation	Base ink for antibody	Surface attachment	References
Pre-activated nylon/membrane	C-reactive protein	Piezoelectric	0.1 M Tris-HCl, 30% sucrose, 0.15 M NaCl, 0.5% PVA, 0.25% Tween 20	Covalent	[79]
Avidin-coated glass slide	Cholera toxin, S. enterotoxin B, ricin, B. globigili	Thermal	10 mM Phosphate buffer, 10 mM NaCl, 10 mM sucrose, 0.1% bovine serum albumin (BSA)	Molecular recognition	[85]
Filter paper	Human and mouse IgG	Piezoelectric	Water	Adsorption	[86]
Glass slide	Cytokines, breast cancer biomarkers, cancer-related protein	Piezoelectric	0.65% Alginate solution, 10% glycerol	Entrapment	[82]
Cyclic olefin copolymer	Procollagen type I C-peptide	Piezoelectric	10 mM Phosphate buffered saline (PBS), 1% BSA	Covalent	[87]
Nitrocellulose-coated glass slide	Rabbit IgG, goat IgG, chicken IgY, human IL-4	Piezoelectric	0.05% Triton X-100	Adsorption	[84]
Nanofibrillated cellulose film	Human IgG	Piezoelectric	10 mM Phosphate buffer	Covalent	[88]
Cyclic olefin copolymer	Calprotectin	Piezoelectric	Phosphate buffer	Covalent	[89]
Cyclic olefin copolymer	Interleukin-6, tumor necrosis factor α	Piezoelectric	Phosphate buffer	Covalent	[90]
Poly(methyl methacrylate)	C-reactive protein	Piezoelectric	PBS, 0.1% Tween 20, 10% sucrose	Covalent	[91]
Nitrocellulose membrane	Pathogenic DNA	Piezoelectric	PBS	Adsorption	[92]

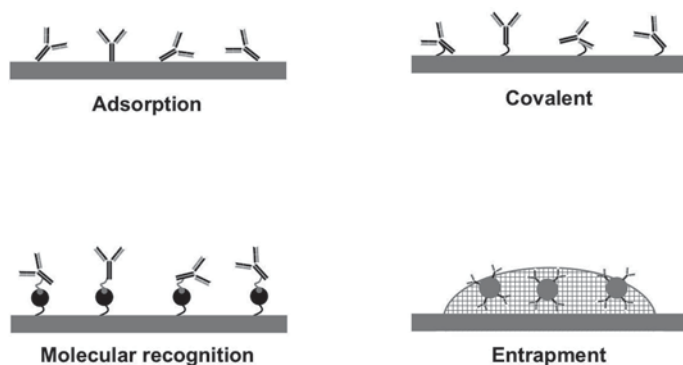


Fig. 8.14 Different types of immobilization for antibodies dispensed by inkjet printing

hydrophobicity on the spot morphology of inkjet-printed biotinylated IgG on both substrates [81]. For this purpose, they have treated the surface with various silanization reagents to vary the contact angle (θ). Based on a nucleic acid immunoassay for the detection of *Staphylococcus aureus*, excellent spot uniformity and good signal-to-noise ratio were achieved with contact angles of $\theta \sim 65^\circ$ and $\theta \sim 75^\circ$ for untreated glass and polystyrene, respectively. On the contrary, to improve the binding capacity, assay sensitivity, and mass transport, 3D porous network materials have been exploited. In this case, the porosity of the material creates an opportunity for short diffusion path lengths and fast interaction between biomaterials. Such work has been demonstrated by Li et al., where they created a 3D antibody microarray by physical entrapment of the antibodies within a highly porous alginate gel that is spotted on a glass slide [82]. This was achieved through sequential reagent printing using an inkjet printer. Nitrocellulose membranes, on the other hand, are widely used porous materials for biochip fabrication. This material has a successful history of use as an adsorbent for Western-blot analysis which allows the measurement of antibody–antigen interactions [83]. Therefore, such application could easily be adapted to antibody microarray fabrication. Commercially available nitrocellulose coated-glass slides have been used to print a NanoProbeArray by a piezo-actuated inkjet printer for the analysis of ultra-low volumes of protein samples [84].

Microfluidic technology plays an active role in influencing a change in antibody microarray devices from a slide format to a microchip format. The microfluidic chip could offer minimal consumption of samples and reagents, short analysis time, integration of other operational steps, and facile fluid handling [93]. The antibodies could be printed in the microchip channels (millimeter length, millimeter to sub-millimeter width, and micrometer height) of various polymeric substrates such as filter paper, COC, and poly(methyl methacrylate) (PMMA). Abe et al. pioneered the fabrication of microfluidic paper-based analytical devices by inkjet etching [94]. Basically, the paper substrate is coated with polystyrene, and the hydrophilic channel is created by etching away the polymer using toluene as the ink. Then, the antibodies are inkjet printed on two different zones of the paper microfluidic immunosensor to form the control and test lines [86]. Figure 8.15 shows the simple

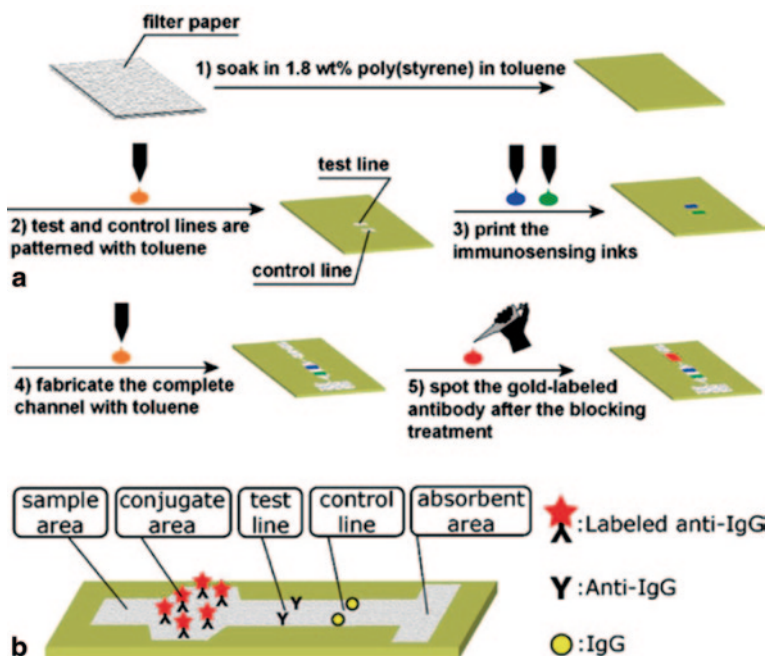


Fig. 8.15 **a** Fabrication process of an inkjet-printed microfluidic immunosensing strip. **b** Schematic representation of the finalized strip. With kind permission from Springer Science+Business Media: Abe et al. (2010) Inkjet-printed paperfluidic immuno-chemical sensing device. *Anal Bioanal Chem* 398:885–893 (reference [86])

fabrication concept of the microfluidic paper-based immunosensor and the deposition of antibodies. This approach has been used for simultaneous multianalyte sensing of two different antigens and pH. Successful demonstration of a qualitative sandwich immunoassay was achieved by showing the difference in response of the test and control lines after sample introduction. Inkjet printing of antibodies on microchannels of nonporous polymer substrates has also been demonstrated. In this case, the mode of antibody immobilization is covalent. This is due to the fluid flow characteristic of the microchip and the absence of a 3D network where antibodies could anchor. Therefore, a wash-stable antibody pattern on polymer substrates is desirable. COC has been used as a substrate for a microchip immunosensor array with antibodies deposited through inkjet printing [87, 89, 90]. Surface modification of COC was done by treating it with p-nitrophenyl ester, which can covalently bind with the amino group of the antibody. Moreover, an aldehyde-functionalized PMMA surface has been used as a substrate for an inkjet-printed antibody. The surface functionalization of PMMA has been achieved through sequential treatment with plasma, polyethyleneimine, and glutaraldehyde (Fig. 8.16). The aldehyde reacts with the primary amine of the antibody to form a stable covalent bond on the surface of PMMA. This surface modification strategy is quite common for antibody immobilization.

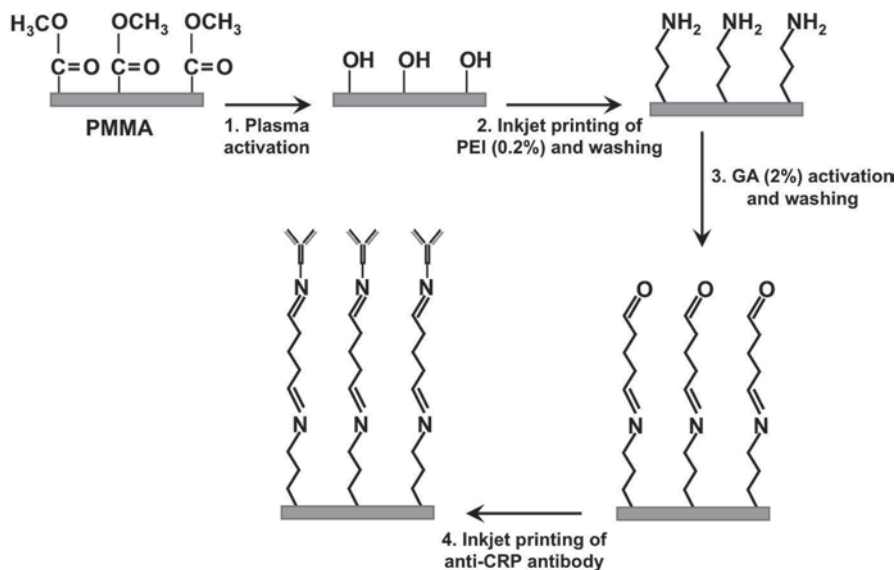


Fig. 8.16 Covalent immobilization of an inkjet-printed antibody array on poly(methyl methacrylate)

8.5 Summary and Future Trends

Starting from around 2000, scientific reports of inkjet printing applied for the deposition of biomolecules with the purpose of fabricating analytical devices based on biorecognition are becoming increasingly numerous. Some applications of inkjet printing of biomolecules have matured close to industrial routine, but others are still in their infancy. While some systems are very robust and reliable for everyday use, others require a highly controlled laboratory environment.

Inkjet printing of nucleic acids has so far been explored mainly for the purpose of DNA microarray fabrication and most attention has been paid to obtain high-quality and high-density arrays. The application of the inkjet technology for the fabrication of other types of analytical devices is still comparatively scarce, despite the method's high potential. Now that manufacturing of DNA microarrays by inkjet printing has evolved into an almost mature technology already commercialized by some manufacturers, the inkjet printing of nucleic acids can be expected to become increasingly applied for the development of other miniaturized analytical tools. Especially miniaturized sensing devices, which enable cost-reduced analysis from low sample volumes, will be of great importance in various fields such as medical diagnosis, and food quality or environmental monitoring, among others. Inkjet printing technology is a prospective approach to handle small amounts of DNAs with the precision required for developing such devices, but at the same time at lowest possible costs.

The inkjet printing of proteins such as enzymes and antibodies on various polymeric surfaces to form selective biorecognition elements is another important

technology that may have high societal impact. It has the potential to facilitate efficient and effective advancements in different areas of research for instance medical and clinical diagnostics, food science, and environmental analysis, among others.

The deposition of enzymes on polymer surfaces by inkjet printing is still considered to be in its early development. This can easily be concluded from the type of examples discussed in this text (e.g., Table 8.4), where most of the enzymes used were either GOx or HRP, which are considered to be comparably stable and widely applied “model enzymes.” Currently ongoing research work is still mainly focused on the effect of inkjet actuation and ink formulation on the activity of the enzyme. A good balance between these two inkjet parameters is essential for a successful enzyme immobilization. Keeping the enzyme in its active form is a major consideration to further develop other immobilization strategies. Given the fact that digital inkjet printing itself is an almost mature technology and that the basic factors regarding enzyme handling by inkjet methods are mostly understood as shown by various research reports, it is apparent to print other important enzymes with relevance to the above-mentioned research areas which could become a significant direction for future efforts. Allowing this development to be driven by the requirement for the analysis of real samples from hospitals, food markets, rivers, and other relevant sources will further push the exploitation of inkjet technology towards more practical applications.

Antibodies are more rigid proteins than the enzymes, since they do not rely on the fragile active catalytic site, but rather on polypeptide chains linked by disulfide bonds. Most of the research work demonstrated is related to the antibody immobilization on various polymeric supports. A key to antibody immobilization is to allow its proper orientation for an effective immunoreaction. This can be achieved by either covalent or biomolecular recognition immobilization strategies. It may be concluded from the examples provided in this text (e.g., Table 8.5) that many of the listed applications target the analysis of disease biomarkers. Those are generally more relevant analytes than the ones addressed by inkjet-printed enzyme-based systems, which probably indicates that the inkjet deposition of antibodies is already in a more advanced state of development.

In general, many recent research reports point to a continuing trend to move from a slide format of assay to a microfluidic chip format. The combination of inkjet printing and microfluidic technologies could certainly lead to more efficient and cost-effective fabrication of a broad range of microfluidic analytical devices. In terms of polymeric substrates applied for these purposes, cellulosic paper and COC seem to be most promising at this time.

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