

Biosensors with Printed Active Layer

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Biosensors are thriving in the detection and precise quantification of a plethora of analytes ranging from water contaminants to biologicals inside the human body. This kind of bioelectronic device has at its heart a thin film with a biomolecule in its composition (active layer), which interacts with the sample to be analyzed and confers specificity and sensitivity to the devices. Here, we review the use of printing techniques to deposit the active layer, emphasizing the advantages and remarkable outcomes of this procedure. After an initial introduction of the main concepts, we discuss the most important aspects of the formulation of bioinks suitable for printing bioactive films. It is stressed that a proper choice of additives is vital for both attaining good printability and preserving functionality of the biomolecules. Moreover, printing conditions must be controlled to avoid further damage to the biological materials. The choice of the printing method depends on the requirements for the target device architecture. For instance, inkjet printing is the method of choice when high resolution features are sought. However, for fast, high throughput fabrication of large-area devices, roll-to-roll (R2R)-compatible mastered methods from the graphics industry (flexography and gravure) are more adequate. Several examples of application are given, and the advantages of using a specific printing method are highlighted in terms of devices performance. Overall, our goal is to illustrate how printing is versatile and effective to fabricate high-end devices through simple and technologically viable processes.

Keywords: Bioelectronics, Biosensors, Biomolecules, Printed Electronics, Inkjet, Rotogravure

1. INTRODUCTION

1.1 Printed Electronics and Printing Techniques

The discovery of conjugated organic polymers in 1977 by Shirakawa and co-workers [1] led not only to the Nobel prize in chemistry in the year 2000, but also to a new era in electronics. These new materials may have its electrical conductivity tuned from null to 10^7 S/m by doping processes similar to the ones used for silicon. However, instead of being insoluble and demand harsh processing methods as ion implantation, etching and e-beam lithography, polymers can be patterned by solution-based processing methodologies. This renders an alternative to silicon which, although still underperforming in comparison to the well-established inorganic semiconductors (and maybe never will be comparable), offers important advantages such as mild processing conditions and possible use of plastic substrates to produce flexible/malleable electronic devices.

It was not too long after the discovery of conducting and semiconducting polymers that scientists and technologists realized printing could be used to deposit patterned constructs of these materials, in an assembly like the one of a transistor. In fact, from the fabrication point of view, that is exactly the very basic task for building electronics: to stack materials in a patterned way; and printing techniques do the job for centuries, depositing solutions of soluble materials (inks) in pre-defined places on a surface. The solution-processability of soluble electronic materials has made this technology viable, and now printing is used to fabricate organic light-emitting displays (OLEDs), organic photovoltaic cells (OPVs) and organic transistors [2].

Printing techniques in general can be classified based on their image carrying medium (ICM), which is the element that stores all the information needed for the printer to distribute the ink at the right places and reproduce the desired pattern. In this context, there are two categories of printing methods: the so-called conventional methods and the non-impact printing (NIP) methods. In the first category ICM is a hardware, often called “master”, with the screen used in the screen-printing technology being a well-known example. Other methods in this category are letterpress printing (flexography), gravure printing and offset printing. The main feature they share is an outstanding efficiency for fast and large-area replication of the printing pattern.

NIP techniques constitute a family of methods that include electrography (toner printing) and photography (color-sensitive coating), with the inkjet technique being by far the most diffused one. Inkjet can be operated in the continuous or drop-on-demand modes, and printers jet the ink based either on the movement of a piezo-driven element or on the thermal expansion of a solvent bubble generated by heat inside an ink compartment. As the name suggests, there is no impact of any printer part on the substrate (no master).

ICM is a software element, like a bitmap, that informs the exact X and Y coordinates for ink dispensing. Inkjet printers are virtually present in any house or office around the world, and can now be an option for mass-production in industry following recent developments of inkjet instrumentation. However, differently from conventional printing methods, the advantage of NIP technologies is not fast replication but rather high precision in ink placement, high resolution of printed features, low-volume of ink used, and no waste of material.

A classification scheme for printing methods based on ICM is depicted in Figure 1, while a more detailed description of printing technologies can be found in ref [3]. The choice of a technique for fabricating an organic (bio) electronic device by printing has to be oriented by a good match between the characteristics of the techniques, and the targeted architecture and process flow.

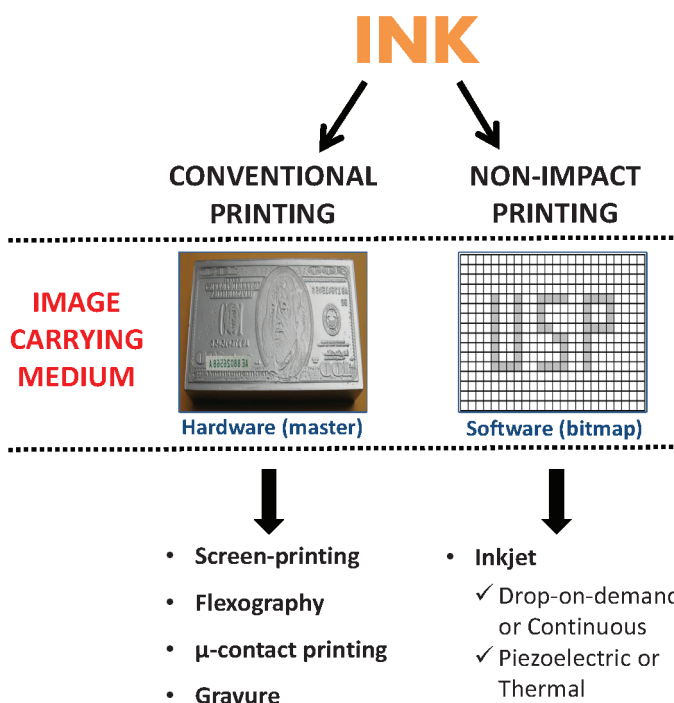


FIGURE 1
 Printing techniques classification scheme, based on the image carrying medium. Listed at the bottom of the picture are only the methods in each category that are used for the printing of active layers in biosensors. A more detailed scheme listing all the printing techniques in each category is given in [3].

1.2 Biosensors

A biosensor is a kind of sensor that has a biomolecule in its recognition element. The biosensor structure, sketched in Figure 2, is basically composed of a transducer interfacing the aforementioned recognition element. While the latter makes contact with the sample to be analyzed, the first is in the opposite side of the structure, frequently connected to a user interface (computer + software) through some wiring or an amplifier.

The function of the recognition element is to interact with the analyte in a specific way that leads to a change in a physical or chemical property of that layer. The transducer in turn translates that change in a measurable signal, which can be optical, electrical, thermal, acoustic, piezoelectric or electrochemical in nature. While transducers are usually solid materials, e.g. metals, glasses, ceramics or plastics, the biomolecules in the recognition elements are soft bioactive entities such as enzymes, proteins, antibodies, antigens and nucleic acids. The way these biomolecules are immobilized on the transducer surface can vary, as will be discussed in this review for cases where printing is employed.

1.3 Challenges of Printing Biomolecules

The task of making inks of electronic materials has been addressed by several researchers and companies around the world, with rewarding results. Conducting and semiconducting polymers have been chemically designed to afford solubility in a variety of solvents [2,4,5]. Moreover, post-drying characteristics of the materials, including molecular packing, crystallization,

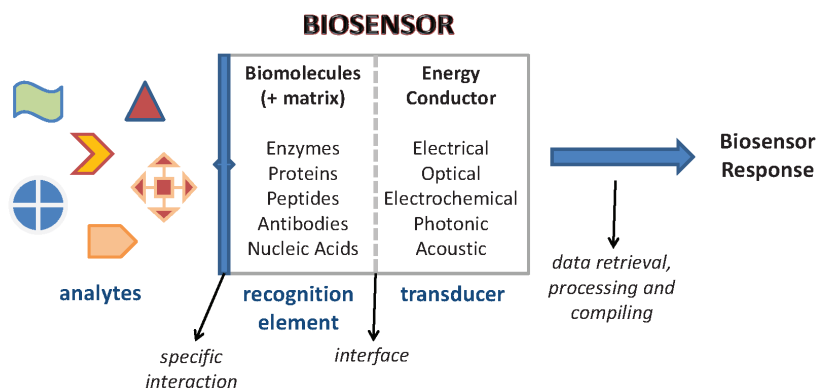


FIGURE 2

Sketch of a biosensor showing recognition element and transducer as main constituents. The recognition element is the active layer that contains the biomolecule for specific interaction with analytes. In a few cases this layer is fused with the transducer, and both structures can be printed. Even analytes are dispensed in some cases by some sort of printing technique.

phase segregation and carrier mobility, are increasingly improving. Strategies such as the use of mixed polymer-small molecule and hybrid organic-inorganic inks are being used to render better printing and devices performance [6,7]. Metal-based inks, in particular, are becoming an alternative for polymers at printing conducting traces, with several commercially available inks already in the market [8].

In printed bioelectronic devices (for instance, a biosensor), transducers can be printed with metal-based or conducting polymers-based inks. This has been done by several authors, especially using screen-printing for electrodes in electrochemical devices [9,10]. For printing the active layer, on the other hand, inks have to be formulated with a biomolecule in its composition, which can be challenging. The latter involves the choice of additives that make an ink amenable for the targeted printing process, without degrading the biological function of the active element. Usually the ink solvent is limited to aqueous buffers and pH must be controlled, and this is critical for the choice of additives. Moreover, the nature of the physical and chemical interactions between biomolecules and ink additives must be considered, in order to avoid or minimize deleterious events, including active site blocking, change in configuration and denaturation, which may kill the recognition event [11].

Besides the intrinsic biological activity of the biomolecules in the ink formulation, another point of concern is the effect of printing conditions over the active element function [12]. In some methods the ink is subjected to harsh processing conditions, including high shear forces, impact and high temperatures. Examples of these situations are the passage through the nozzle of the inkjet print-head, the jetting of ink in thermal ink-jetting, and blade sweeping in rotogravure or screen-printing. The amount of energy dispended in such mechanical and thermal events as well as its effect over the biomolecules must be estimated to predict possible damage to the material. Ideally, the biomolecules should be able to withstand such conditions by themselves or with the help of additives that may absorb part of the energy.

There has been significant efforts worldwide to overcome most of the problems mentioned above (see [12,13] for a detailed discussion), and bio-printing is already a reality. Proteins and cells have been ink-jetted and 3-D printed; printing constructs used in tissue engineering and in building artificial organs have been reviewed in [14]. Here, we firstly discuss bioinks specifically designed for biosensors (section 2), emphasizing the strategies used in this field to overcome the challenges mentioned. Subsequently, the use of printed bioactive films as active layers in biosensors is reviewed. The biosensing devices are classified in two broad groups, depending on the process to generate the active layer: printed by inkjet or by large-area methods. These are the subjects of sections 3 and 4, respectively. Although some patents are cited along the text, we restrict this review mostly to the academic literature.

2. BIOINKS

The nature of the events causing stress to biomolecules depends on the printing methodology. For instance, inkjet relies on the passage of ink through the nozzle orifice, and the fluid is accelerated causing an increase in the shear stress. Furthermore, ink has to be compressed inside the nozzle compartment to be expelled, and this can be fairly aggressive to biomolecules. Specifically in piezoelectric-driven inkjet, the mechanical stress can be regulated by the waveform and voltage applied to the piezo element. Nishioka et al. [15] were the first to systematically study the effects of piezoelectric inkjet printing parameters over the activity of an enzyme. They showed that the shorter the duration of a fixed voltage applied to the printing head the higher the extent of degradation for the peroxidase enzyme. Hence, the degree of damage is reduced if actuation is slowed down, and the residual enzyme activity can be even kept unchanged if printing is performed at compression rates slower than $2.5 \times 10^4 \mu\text{m}^3/\mu\text{s}$. Similar findings were reported later by Yan et al. [16], who also showed that additives as trehalose can stabilize enzymes for printing at higher compression rates (see discussion below).

Brian Derby's group at The University of Manchester has proven a few years later that piezoelectric inkjet of enzymes can be performed with no significant damage to the biomolecules [17,18]. They conducted an extensive study of the effect of jetting parameters on the activity of glucose oxidase (GOx), and showed that although the concentration of enzyme in distinct jetted droplets can change, the chemical integrity of the enzyme is preserved. Data from size exclusion chromatography, light-scattering and circular dichroism measurements, reproduced in Figure 3, show that molar mass, hydrodynamic ratio and tertiary structure for GOx are not altered after piezoelectric inkjet printing. Furthermore, more than 70% of the initial enzymatic activity was preserved for any combination of actuation parameters (voltage and waveform). The applicability of piezoelectric inkjet to other enzymes was further confirmed by different authors (examples in section 3), with the requirement of avoiding high voltages in some cases [19].

As pointed out in section 1.3, additives are usually incorporated into the solutions of biomolecules to turn them into inks (printable fluids). However, it is important to guarantee that no deleterious effects are imposed to the function of the bioactive materials. Ness and co-workers [20] discussed a few challenges for the formulation of a bioink. Consistent with the rationale presented here, they stated that factors influencing the fixation and biological activity of the biomolecules, e.g. salt concentration, solids concentration, solution evaporation rate and pH, must be tuned at the same time that properties relevant to jetting (viscosity and surface tension) must fit into an adequate window for the printing system to be used.

Yan et al. published in 2007 a seminal paper addressing the effect of viscosity modifiers and surfactant (the two most important additives for inks)

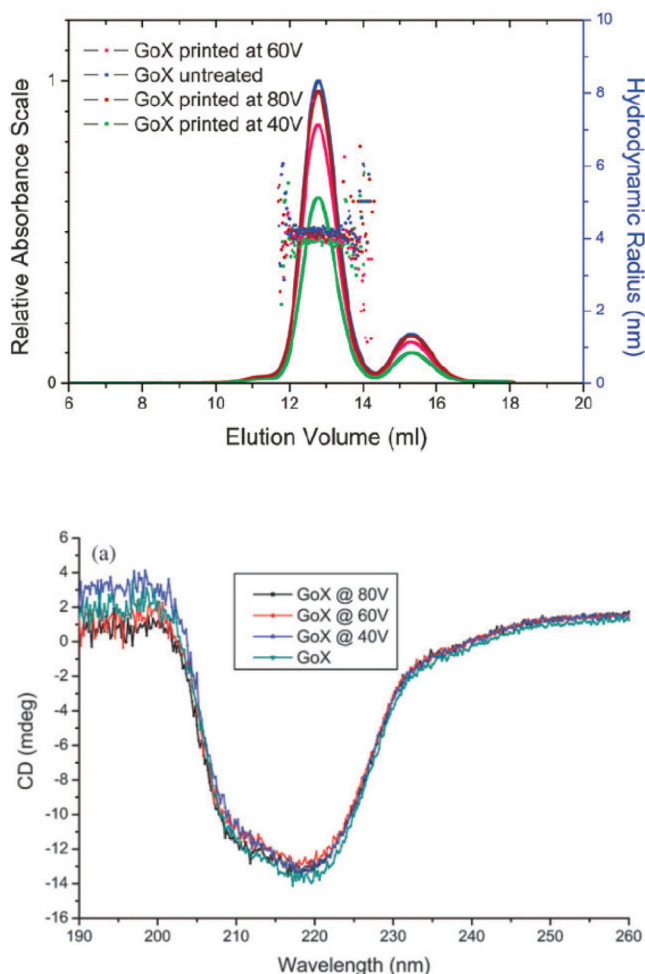


FIGURE 3

Top: Light scattering intensity and derived hydrodynamic radius measured for non-printed GOx solution (untreated), and GOx solutions printed at three different piezo voltages [Reproduced with permission from [17]. Bottom: circular dichroism spectra for a native GOx solution (GOx), and GOx solutions ink-jetted at three different voltages (40, 60 and 80 V) [Reproduced with permission of The Royal Society of Chemistry, doi: 10.1039/c0cc00567c from [18]]. Overall, the enzyme had its conformation and tertiary structure roughly unaffected by the piezoelectric inkjet process.

over enzyme activity [16]. Using horseradish peroxidase (HRP) as a model enzyme they proved that Triton X-100[®] is inoffensive to the biomolecule in concentration lower than 0.05 wt% in a phosphate buffer pH 6.8. This concentration is above the critical micelle concentration (0.02 wt%) of the surfactant, and therefore its ability of decreasing surface tension can be used at most [17]. As a rule of thumb, non-ionic surfactants are preferred for bioink

formulations, since most biomolecules are charged (e.g. enzymes) and can be degraded or even denatured by ionic detergents via electrostatic interaction and formation of charged complexes. However, specific non-ionic surfactants such as commercial Brij 35[®] can cause “side-effects”, e.g. excessively quick drying of inks leading to nozzle clogging [20]. In general, Triton X-100[®] and surfactants from the Tween[®] family are used in the literature.

Regarding viscosity modifiers, Yan and co-workers compared the power of the most common thickeners used for ink-making, as well as their effect over HRP catalyzing activity. The results are reproduced in Figure 4. As expected,

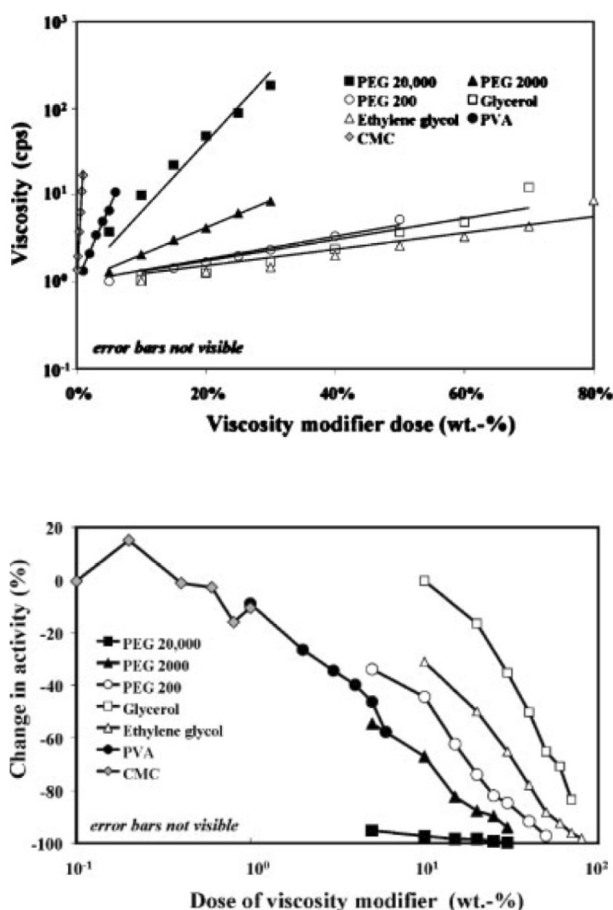


FIGURE 4

Top: Dependence of solution viscosity on concentration for the materials most commonly used as viscosity enhancers in biological and electronic inks. Bottom: change in HRP activity caused by the same materials in the same range of concentrations [Reproduced with permission of John Wiley & Sons, Inc. doi: 10.1002/marc.200700226 [16]]. All the solutions in both graphs were made in 0.04 M phosphate buffer (pH 6.8) and contained Triton X-100[®] in the concentration of 0.1 wt%. HRP concentration for measuring enzyme activity was 5×10^{-4} M.

polymeric thickeners are more efficient in augmenting the viscosity of the ink, and their efficiency in doing so increases with increasing molar mass. However, high molar mass polymers are more prone to cause damage to enzymes, as seen in the poly(ethylene glycol) (PEG) analogous series, where PEG 20,000 almost completely inactivates HRP. The authors speculate this is due to the limited diffusion of enzyme substrates and products of the enzymatic reaction in high molar mass polymer solutions. Curiously, charged polymers such as CMC are the most efficient viscosity enhancers and cause the lower degree of damage to the enzyme. It is suggested that the contribution of the polymer charge density to the increase in viscosity for these polymers prevents overcrowding of the ink, helping the biomolecule to perform better. Glycerol was added as humectant to the ink to avoid first drop ejection problems caused by solvent evaporation at the nozzle. With optimized conditions the loss of HRP activity was less than 2%.

Although enzymes at first glance appear to be very susceptible to damage by having a complex 3-dimensional structure sustained mostly by weak interactions such as hydrogen bonds, from the bioink literature these molecules are considered suitable also to printing methods involving harsher conditions than the ones found in piezoelectric inkjet. For example, thermal inkjet is supposed to involve much more aggressive conditions, since ink expelling from inside the nozzle is driven by the collapse of a thermally generated pressure bubble inside the printing head. Despite that, thermal-based office inkjet printers have been used to deposit enzymes like β -galactosidase, with the loss of original enzymatic activity reported to be only 15% [21]. It is speculated that although local heating of the ink is in the order of 200-300 °C, its short duration (around 2 μ s) together with heat dissipation in the ink volume inside the nozzle cause the portion of fluid to be dispensed not to be heated at all. Furthermore, additives such as glycerol play a stabilizing effect, and thermal inkjet is described for other enzymes as well [22,23].

Other examples of enzyme printing under harsh conditions but with no serious damage come from Talbert et al [24] and Jabrane and co-workers [25]. In the first [24], GOx was made soluble in toluene by ion pairing with the surfactant didodecyl- dimethyl-ammonium bromide (DDAB). This allowed for better spreading on hydrophobic plastics and enable alternative applications, for instance, in packaging. Despite a huge degradation of enzyme activity to less than 1% due to enzyme modification and inkjet-printing, glucose could still be colorimetric sensed with printed films. In [25], the authors employed HRP in rotogravure printing and, again, there was colorimetric evidence of the residual catalyzing effect of the biomolecule. The enzyme resistance to the tough gravure conditions was predicted by theoretical calculations, according to which the stress experienced by Newtonian inks in the rotogravure process with a typical shear rate of 10^6 s^{-1} (having water as solvent - viscosity $\sim 1 \text{ cP}$) is around 1 kPa, but the share of this stress effectively transferred to a typical globular protein of (roughly) 10 nm in

diameter is just around 100 pN. If enzymes are approximated to be ideal linear polymers, these forces could be sufficient to break intramolecular bonds and stretch the molecules. Nevertheless, the relaxation time for them to recover the original structure would be in the order of tenths of a μs , and the biomolecules could possibly stand the process without being damaged (as observed in the experiments).

3. BIOSENSORS WITH INKJET-PRINTED ACTIVE LAYER

The use of inkjet for deposition of the active layer in biosensors is a growing field. Komuro et al. [26] recently reviewed (bio)chemical sensors where inkjet was used to deposit some layers of the device (electrodes, biological or non-biological active layers, etc). The authors offer an interesting comparison of inkjet with other fabrication methods for biodevices, but their scope (in their words) extends to the whole family of “sensors” or “sensing devices”, not only to biosensors. Gonzales-Macia et al [27] conducted an even broader analysis of deposition methodologies (including printing) for fabrication of biodevices (including biosensors). In the present section of this review, we narrow down the scope and focus on biosensors and on inkjet printing of biomolecules for use as recognition element. Special attention is given to the advantages offered by the inkjet methodology from the fabrication point-of-view.

To the best of our knowledge, enzyme inkjet was first reported by Kimura et al in 1988 [28]. They used inkjet for the localized dispensing of GOx and Urease on a Si-based ISFET device, which was employed to detect glucose and urea. Bovine Serum Albumin (BSA) was added as stabilizer to the inks. Enzyme fixation was attained by cross-linking with glutaraldehyde either using liquid or vapor-phase reactions, with the second methodology leading to more uniform films. Four years later Newman and co-workers [29] ink-jetted GOx on top of conventional electrochemical glucose strips composed of screen-printed electrodes, in an first attempt to fabricate an all-printed device (the redox mediator was also printed). Several passes of the printing head helped improve biosensor performance, reproducibility was high (within 5% of compliance) and the response time was always lower than 1 min. In the following years, the use of inkjet to dispense biomolecules and the formulation of bioinks have grown to a point where technologically viable products were conceived and patented by companies like Canon [30,31] and Roche Diagnostics [32].

Table 1 summarizes the papers in the literature describing inkjet for depositing the active layer of a biosensor. The printed biomolecule, ink additives, biosensor target analyte and detection method are listed. It is worth noting that we only included studies in which ink formulation is described in detail and quantitative biosensing was performed. Other relevant papers are discussed in the text.

TABLE 1

List of papers from the literature describing the use of inkjet for depositing the active layer of biosensors. Only works in which ink formulation and detection measurements (including analytical curves) are described in detail were included.

Printed Biomolecule	Ink Additives	Target Analyte	Detection Method	REF
GOx	no additives	glucose	Electrochemistry	[29]
GOx	EDTA and glycerol	glucose	Electrochemistry	[22]
GOx	Vinnapas [®] EP16 (vinyl acetate and ethylene copolymer)	glucose	Scanning electrochemical microscopy	[33]
GOx and HRP	o-toluidine and L-ascorbate	glucose	Colorimetric	[34]
GOx and HRP	PEDOT:PSS	glucose	Electrochemistry	[35]
GOx or HRP	poly(pyrrole) nanoparticles	glucose or H ₂ O ₂	Electrochemistry	[36]
GOx or Urease	BSA	glucose or urea	Transistor-based	[28]
HRP	SDS	H ₂ O ₂	Chemiluminescence	[37]
HRP	EDTA and glycerol	H ₂ O ₂	Electrochemistry	[23]
HRP	3',5,5'-tetramethylbenzidine dye and glycerin	H ₂ O ₂	Colorimetric	[38]
Urease	glycerol and Triton X-100 [®]	urea	Electrochemistry	[39]
Tyrosinase	no additives	bisphenol A	Colorimetric	[40]
Acetylcholinesterase	glycerol and Triton X-100 [®]	organophosphates (neurotoxins)	Colorimetric (Ellman Assay)	[41]
Acetylcholinesterase	glycerol and Triton X-100 [®]	acetylcholinesterase inhibitors (pesticides)	Colorimetric	[42]
b-galactosidase	not reported	heavy metals	Colorimetric	[43]
b-galactosidase	no additives	E.Coli and B. Subtilis bacteria	Colorimetric	[44]
BSA-mannose, BSA-galactose, BSA-OEG, RNase B	no additives	GRFT and RCA lectins	Photonic (microring resonator)	[45]
DNA-based oligodeoxyfluoroside (ODF) dyes	polyethyleneglycol	volatile sub-products from food spoilage and ripening	Fluorescence	[46]
Analyte-specific biotinylated antibodies	sucrose and BSA	cholera toxins, staphylococcal enterotoxin B, ricin and Bacillus Globigii	Fluorescence	[47]

Analyte-specific biotinylated antibodies	EDTA and Tween 20 [®]	urokinase-type plasminogen activator and its type-1 inhibitor (cancer markers), and vascular endothelial growth factor	Fluorescence	[48]
BSA, BSA-phenytoin conjugate, biotinylated-HRP, biotinylated-BSA, biotinylated alkyl thiol	no additives	anti-BSA and anti-HRP	Surface plasmon resonance	[49]
anti-PICP (procollagen type I C-peptide) antibody	no additives	PICP	Luminescence imaging	[50]
anti-humanMRP8/14 and S100A8/A9 antibodies (monoclonal anti-calprotectin antibody)	no additives	calprotectin protein	Chemiluminescence (SuperSignal [®] WestFemto commercial kit)	[51]
mouse anti human IgG	no additives	human IgG	Optical reflectivity	[52]
biotinylated-BSA antiserum Ab2114 and coating antigen (cAg) 5cona, antiserum Ab4652 and cAg 7, Ab245and cAg 7b-cona	glycerol and Tween 20 [®] or Triton X-100 [®]	streptavidin (proof-of-concept)	Fluorescence imaging	[20]
	ethylene glycol	hydroxyatrazine, carbaryl and molinate	Fluorescence	[53]

From a quick inspection of table 1, a few trends in the field can be noticed. Firstly, glucose and H₂O₂ are still the major target analyte, and GOx and HRP are the enzymes most common in bioinks. This is obviously due to the high relevance of these analytes in view of biomedical applications, and because there is considerable literature for these analytes and enzymes which facilitates comparison. Secondly, the interest in printing antibodies for construction of immunoassays is remarkable. The latter is possibly owing to the requirement of precise placement of biological materials, e.g. in the parallel detection of multi-analytes [49], discussed below. Finally, in terms of bioink formulation, there is predominance of the additives Triton X-100 and EDTA.

However, the printing of pure solutions of biomolecules is also possible when office printers [44] or dispenser-like printers are used [50,52], and when high resolution features are not a requirement [29,40].

Immunoassays have been produced by inkjet in different geometries, including disposable cross-hatch structures with two enzymes intercalated on a nylon membrane [54], spotted arrays on a CD-disc [53], and as Y-shaped microfluidic devices with embedded photonic crystals and antibodies [52]. Printed immunosensors find such innovative applications as determination of blood type by systems printed on paper [55], selective detection of pesticides by multiplexed biosensors [53], and detection of malaria disease [56]. In addition, the inkjet capability of accurate ink placement is explored to pattern multiple antibodies in a single micro-device [49], and to integrate antibodies to microfluidic chips [57]. In general, loss of activity/specificity for this class of molecule after ink-jetting is not reported, indicating the molecules are very stable to the process. Moreover, although ink formulation and viscosity are rarely optimized, they seem to have adequate jettability using most of the printers available for research.

A specific kind of immunoassay for which a printed version is being pursued is in ELISA-like assays (enzyme-linked immunosorbent assay). For instance, Yatsushiro et al [50] reported the inkjet fabrication of a microchip to detect the carboxy-terminal peptide of type I procollagen, which is an indirect way to monitor the rate of type I collagen production in the body, and hence bone formation. Almost a decade earlier, Delehanty and co-workers had already shown that similar flow-based devices loaded with inkjet-printed biomolecules had performance comparable to the conventional ELISA method, but with improved sensitivity [47]. This is an interesting demonstration of how printing can be used instead of more complex methods to fabricate functional devices.

Another tendency in the use of inkjet for biosensors arises from the fact that the technology is fully adapted to printing on paper, by far the most commonly used substrate. Devices based on bioactive-paper have the advantage of being cheap, light and amenable to home-fabrication. For instance, Baraldini et al. [37] used a commercial office printer (HP DeskJet 600) to deposit bioactive microarrays on paper. Black ink cartridges were cleaned and re-used for the uniform deposition of periodically distributed HRP dots, with enzymatic activity among the dots varying by less than 10% (within 100+ spots). Additional strategies can now be used to make the fabrication process even more technologically straightforward. For example, Daniel Citerio's group in Japan has fabricated (with an office printer) an all-printed microfluidic device on paper through a three-step additive process based on the photocuring of a hydrophobic acrylic resin [38]. The authors built upon another all-inkjet-printed device [34], where inkjet etching had been used to pattern polystyrene channels on paper, followed by active layer multi-pass

inkjet deposition. The devices were employed to detect glucose and H_2O_2 in biologically relevant concentrations.

In order not to compromise the simplicity of paper-based devices, colorimetric biosensing is usually performed, either by monitoring color change due to generation of sub-products of the biosensing reaction [46] or by addition of colored indicators [58]. The latter procedure is also used in biosensors deposited on other kinds of substrates [22,23,38,42]. For paper-based, inkjet-printed devices, parameters such as the spreading and penetration of bioinks in porous papers [58,59], and long-term stability of inkjet-printed bioactive papers, are already well characterized [58,60]. Noble applications, including detection of food spoilage and ripening, have been described [46].

In the last few years, bioink formulations are increasing in complexity, with functional nanomaterials being incorporated to the solutions. The base ink composition can be already more complex, as in a series of paper from Brennan's group at McMaster University, who used sol-gels to stabilize biomolecules and attain good printing [41–43]. Acetylcholinesterase (AChE) and β -galactosidase (β -gal) enzymes were printed in sol-gels, and neurotoxins, pesticides or heavy metals were efficiently detected in liquid samples. A similar biosensor in which microgels containing DNA and antibodies were inkjet-printed was described in a previous work from a different group from the same institution [61].

The conducting polymers PEDOT:PSS, PANI and PPy [22,23,36,39], and Au nanoparticles [44] were also included in bioink formulations and inkjet-printed onto a variety of rigid and flexible substrates to fabricate a biosensor. PEDOT:PSS is especially attractive by being water-soluble and biocompatible. PANI in a nanoparticulate form was helpful at interacting and filtering sub-products of the urease reaction. The enzyme lost less than 2% of its activity, and urea quantification in blood serum showed 85% accuracy when compared with routine clinical methods [39]. Also, the use of nanoparticulated PPy, combined with multilayer printing and co-dissolution with HRP, led to very efficient sensors for H_2O_2 [36], with sensitivity being 3 times the value of similar sensors that were obtained with a single layer of inkjet-printed PEDOT:PSS solutions (not nanoparticles) [23].

To close this section, attention is drawn to a collection of papers that exemplify the fabrication of unique biosensors made possible exclusively by the highly localized and precise dispensing of materials afforded by inkjet. In the work of Creran et al. [44], inkjet was used to deposit patterns of β -gal and a chromophore in the active layer of a paper-based biosensor for bacteria contaminants in drinking water. The flexibility in changing the sort of pattern to be printed (checker, confetti, spheres, etc) helped to tune for higher color change, and hence better visual distinction at detection [44]. In a similar fashion, arrays of growth factors with graded density of the biomolecule were fabricated by different number of overprints, which allowed for cell growth

with controlled concentration [62]. GOx was printed as lines and grids with graded enzymatic activity on an Au surface [33].

Finally, in Kirk et al [45], inkjet was used to functionalize silicon-based photonic microchips (micro-ring resonators) with six types of glyco-bioconjugates, rendering a multiplexed biosensing platform that integrate printed and conventional electronics. A scheme of the final device is represented in Figure 5, where the perfect positioning of the biomolecules on top of the micro-rings is illustrated. In another publication by Arrabito and co-workers [19], the ultra-low-volume deposition capability of inkjet was explored to fabricate a bioarray for drug screening composed of droplets of biochemicals (enzymes, liposome, drugs and fluorophores) layered on the same spot of a Si substrate, with a total final volume of 480 pL (Figure 6). Glycerol (30% v/v) was added to the inks to facilitate jetting and keep the spots wet up to 8 h. A side-effect was the reduction of IC50 values for the assay, which was attributed to slower diffusion coefficients for analytes and reduced protein flexibility in the glycerol medium. The system was employed in the detection and quantification of the inhibitory potential of drugs by a change in brightness, with the reaction taking place inside the volume of each droplet. It is worth

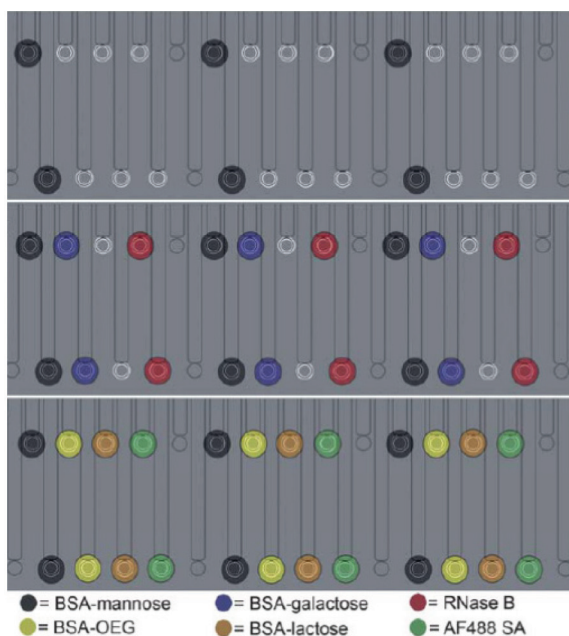


FIGURE 5 Schematic representation of micro-ring resonators fabricated on Si and coated with different biochemicals (BSA-mannose, BSA-galactose, BSA-OEG, BSA-lactose, RNase B and AF488) using drop-on-demand inkjet-printing [Reproduced with permission of The Royal Society of Chemistry, doi: 10.1039/c0lc00313a from [45]].

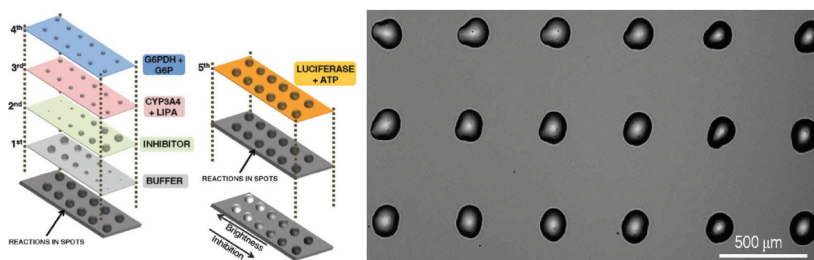


FIGURE 6

Left: process flow for the fabrication of an in-droplet immunoassay biochip through the layer-by-layer assembly of biochemicals using inkjet, and indicative of the detection method through the monitoring of droplets brightness. Right: optical image showing an array of 18 reaction compartments with graded brightness due to a graded concentration of the inhibitor analyte [Adapted and Reproduced with permission of The Royal Society of Chemistry, doi: 10.1039/c2lc40948h from [19]].

noting that inkjet in this case allows for high throughput fabrication of very uniformly distributed reaction vessels, which are also shown in Figure 6.

4. BIOSENSORS WITH LARGE-AREA PRINTED ACTIVE LAYER

The use of conventional (mastered), large-area printing methods for deposition of biomolecules as active layers in biosensors is much more restricted than the use of inkjet, with the exception of the screen-printing method. Screen-printing was first explored in the field and patented in the late 1980's [63], being studied extensively in subsequent years. Over 40 papers dealing with the screen-printing of enzymes are found in the literature. Albareda-Sirvent et al [64] reviewed in 2000 a collection of nearly a dozen papers describing the formulation of bioinks and pastes for screen-printing patterned biologically active films. Two approaches have been used for screen-printing active layers of biosensors. The first consisted in the individual deposition of layers of conductive and biological material stacked on top of each other. In the second, biocomposite pastes have biomolecules and conductors in their composition. A more recent list of works in the area can be found in the Introduction of ref. [65]. A detailed discussion of technical aspects of these works is out of the scope of the present review, and therefore only some advances in screen-printed biosensors will be discussed.

So far, only enzymes have been used in printed biosensors, with the only exception of antibodies employed by Wang et al [66]. GOx is the most studied biomolecule, but AChE, HRP and Lactate Oxidase (LOD) are also extensively explored. In terms of ink composition, graphite(carbon)-based inks are by far the most used formulations, often prepared by a simple mixture of additives to commercially available carbon pastes. Polymers such as PVP are

used to control ink viscosity, and sol-gel materials are also explored. As far as the solvent is concerned, organic-based carbon pastes were historically preferred, but this has changed since the pioneering work from John P. Hart's group [67] that introduced water-based carbon inks, which are less aggressive to both enzymes and environment. More recently, with insoluble electron transfer mediators in the formulation (e.g. CoPC), water-resistant screen-printed active layers for biosensors could be deposited in a one-step printing process [68]. The latter is an advance compared to previous works with aqueous-based inks, which required either a protecting layer deposited in an extra printing step [67] or an extra cross-linking treatment of a UV-polymerizable resin [69].

The use of sol-gel-based inks is another remarkable trend in the field, because these inks can provide improved stabilization of biomolecules [66,70,71]. Maattanen et al. [72] have screen-printed a water-based GOx-containing ink on a paper-based device having hydrophilic wells patterned with flexographically printed PDMS, in an interesting example of a large-area, all-printed colorimetric biosensor. As one should expect, screen-printing of the whole biosensor could lead to a fast, technologically compatible fabrication protocol [73]. Applications reported include analysis of lactate in dairy products, e.g. milk and yoghurt [74–76], of pesticides [77] and other biological molecules in blood serum [68,78].

Another mastered printing technique employed for biosensors is microcontact-printing (μ CP), also known as soft lithography. Though this use started more than 15 years ago [79], and is continuing to evolve [80], only a few works are found for printing the active layers. The method is reported to be more adequate than spontaneous adsorption from solution to deposit individual GOx molecules on a metal surface [81]. However, microcontact-printing is usually applied for the deposition of periodic structures, like strips and gratings with features in the order of about 10 μ m. Nichkova et al. [82] printed such structures with inks of BSA-linked antigens and used them as templates for adsorption of nanoparticles and subsequent detection of phenoxybenzoic acid, a biomarker that indicates exposure to insecticides. Lee et al [83] and Pats-Alfonso et al. [84] published similar works with detection of proteins and cancer biomarkers, respectively. Quantitative analytical curves were reported in all cases. In addition, fluorescent patterned quantum dots (QD)-bioconjugate arrays were transferred for detecting protein binding events [85]. A biosensor for L-Glutamate was fabricated via μ CP of the enzymes glutamate oxidase (GLOD) and glutamic-pyruvate transaminase (GPT) on Ta₂O₅ [86]. Finally, the selective μ CP immobilization of BSA-modified antigens on plastics [87] was extended to produce ELISA-like immunoassays on ITO electrodes coated with a redox polymer [88].

Although screen-printing and μ CP are able to generate replicates of a desired patterned in a simple, fast fashion, these techniques are not easily

adaptable to a roll-to-roll (R2R) process. Therefore, they are not the best choice when real large-area, high-throughput fabrication is pursued. Two other large-area printing methods from the graphics industry started to be explored in recent times for printing the bioactive layers in biosensors: i.e. flexography and rotogravure. Flexography is being exclusively explored by Maria Smolanders' group at VTT (Valtion Teknillinen Tutkimuskeskus) - Finland. They have not actually applied the films for analytical biosensing, but they formulated Laccase-containing bioinks suitable to large-area printing. In their first publication [89], Laccase was encapsulated into PEI capsules synthesized by interfacial micro-emulsion polymerization. The encapsulated enzyme had 70% of its original activity preserved after 5 months stored at 4 °C, compared to 50% for the free enzyme in buffer. A further activity decrease of 30% was seen after mixing the capsules with a typical sulpho-based binder polymer used in flexo-printing inks, but then no extra losses of activity were seen in the following 8 weeks. The ink was printed on paper using screen-printing and flexo, and oxygen consumption measurements demonstrated that the printed films were bioactive.

The same research group used non-encapsulated Lacasse in bioinks for flexographic printing [90,91]. Three commercial binding resins were tested, with the sulpho polyester resin HZ1100D being shown not to be deleterious to enzyme activity even after 30 days of mixing, with better results attained in a slightly basic pH (7-8). Paper samples coated with these inks using flexographic printing showed change in color in the presence of ABTS dye, which is oxidized by Laccase. This unequivocally proves that the enzyme is stable to ink additives and the printing process. The use of the bioactive paper as oxygen indicator in packaging (since there is consumption of oxygen in the enzyme-mediated reaction) or as anti-counterfeiting additive when paper authenticity is key are speculated.

Gravure-printing as a tool for depositing patterned films of biomolecules is the subject of a few patents [92,93], in which new telluride-based ligands for bioinks and a diffraction-based detection method are described. Immunosensors are targeted, but no biosensing is demonstrated. In the academic literature, printing biological materials using gravure was first tried by Jabrane and co-workers [25,94]. The authors commented on the challenges for enzymes to stand the high shear stresses involved in cylinder inking (blade sweeping) and ink transfer (cylinder pressing against substrate) in the gravure method, and even did some calculations of these forces, as commented in section 2. Experimentally, they showed to be possible to print bioactive films of HRP and a bacteriophage (T4), with the same being used in colorimetric detection of H₂O₂ and of living bacteria. As predicted, the catalytic activity of the biomolecules decreased with an increase in ink viscosity or printing speed, since both cause an increase in shear stress. The latter shows that shear stress in gravure does play a role in enzyme deactivation. On the other hand, it also confirms that biomolecules can be printed and stay active. In ref. [94],

the influence of gravure printing parameters on phage transfer was studied. In general, the higher the printing speed and print force the lower the transfer of the biological materials. Moreover, phages concentrations as low as 10^4 are sufficient to attain the same bioactivity found with 10^7 phage concentration in the 2008 paper if higher porosity papers were used as substrates. The bioactive papers so described could be used as biosensors if operational parameters are adjusted.

Another paper describing the printing of bioactive films using gravure (again not used for analytical biosensing) comes from Heikkinen et al [95]. The authors focus on the synthesis of silane-functionalized avidin-containing bioinks capable of adhering on sol-gel-coated PMMA. Silane substitution (methyl or ethyl), avidin concentration and the avidin to silane ratio were varied to obtain inks with better printability and improved fixation. In general, gravure printed films were not homogeneous, with a slight improvement when 0.6 mg/mL of avidin was used instead of 0.3 mg/mL. However, even for the more concentrated ink, viscosity was still too low for gravure printing (lower than 1 cP). The same inks showed to be printable by the inkjet method, with bioactivity preserved even after 2 months of preparation. Bioactivity for the films was demonstrated by measuring the presence of the complementary biotin analyte in the test solution using fluorescence.

Finally, Pavinatto et al [96] described the fabrication of an all-printed biosensor for antioxidants by the combined use of inkjet and rotogravure. Based on bioinks used for inkjet (described in section 3), an optimized selection of additives was employed in a formulation capable of preserving around of 15% of original Tyrosinase enzymatic activity after gravure printing. Ink rheology and surface tension as well as printing parameters (speed and pressure) and substrate surface energy were optimized to obtain patterned and homogeneous bioactive gravure-printed films (shown in Figure 7a). Such films were printed on top of Ag and Au inkjet-printed, high resolution, interdigitated electrodes deposited on plastics (Figure 7b), leading to flexible biosensors. Such biosensors were tested in electrical impedance biosensing of a model antioxidant, showing a detection limit of 200 μM and a linear range up to 1.2 mM. The final architecture and a picture of the biosensor are shown, respectively, in Figures 7c and 7d.

5. CONCLUDING REMARKS

The list of almost 100 papers reviewed in this manuscript (most of them published in the last decade) shows that the use of printing techniques for the deposition of the active layer in biosensors is a hot topic. Similarly to the organic electronics area, printing can lead to innovative processes for biosensors that are compatible with simple and cheap protocols for fabrication of flexible devices. A first and pivotal step for printing the active layer of biosen-

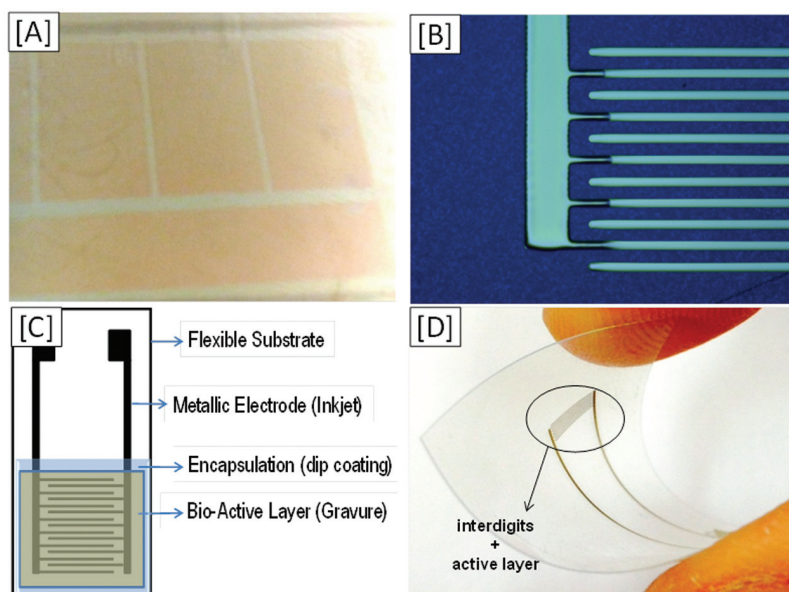


FIGURE 7

(a) bioactive, patterned film of a Tyrosinase-containing ink printed by rotogravure; (b) Au interdigitated electrodes printed by inkjet on a plastic substrate; (c) sketch of the architecture of the all-printed and flexible biosensor; (d) photo of the all-printed and flexible biosensor [Reprinted from [96], doi:10.1016/j.bios.2014.09.039, with permission from Elsevier].

sors is the formulation of bioinks. Even though changes in biological activity were addressed quantitatively only in a few of the works reviewed, knowledge of effects from incorporating additives, and of the printing process itself over biomolecules activity is important to guarantee biological performance. This requires methods to evaluate bioactivity for some classes of molecules other than enzymes (e.g. antibodies, DNAs and peptides). Moreover, in view of technological applications, a more extensive characterization of the long-term stability of bioinks and bioactive printed films still remains to be done for most of the systems.

Inkjet is being heavily used in biotechnology for the fabrication of immunoassays, taking advantage of the very precise placement of low volumes of biomolecules solutions afforded by the technique. Microchips with potential for replacing the ELISA method are being explored, and commercial desktop printers are used for the fabrication of such devices. In conjunction with the widespread use of paper-based devices, colorimetric detection and microfluidics, this can lead to technologically appealing processes for home-printing of biosensors for point-of-care diagnosis. From the scientific perspective, the combination of functional nanomaterials (mainly metal and semiconducting

polymers nanoparticles) with biomolecules in bioinks deserves attention. For this could possibly lead to fabrication of highly efficient, highly specialized biosensors.

Finally, the use of mastered and large-area methods for printing bioinks is growing in recent years, with screen-printing, microcontact-printing, flexography and rotogravure being explored. Ink formulation is especially challenging for these methods, because the mechanical forces involved are usually more intense than in inkjet. If this is successful, however, the reward will be considerable since fast and roll-to-roll (R2R) compatible fabrication of bioactive films can be attained. Then, cheap large-area fabrication of biosensors will be made possible. By judging from cutting-edge works in the field, the ideal and more profitable processes for biosensor fabrication must arise from the combination of inkjet with some of the mastered methods mentioned here, in which advantages would be taken from both classes of techniques.

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