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Microfluidic enzymatic biosensing systems: A review

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ABSTRACT

Microfluidic biosensing systems with enzyme-based detection have been extensively studied in the last years owing to features such as high specificity, a broad range of analytes and a high degree of automation. This review gives an overview of the most important factors associated with these systems. In the first part, frequently used immobilization protocols such as physisorption and covalent bonding and detection techniques such as amperometry and fluorescence measurements are discussed with respect to effort, lifetime and measurement range. The Michaelis–Menten model describing the kinetics of enzymatic reactions, the role of redox mediators and the limitations of the linear measurement range of enzymatic sensors are introduced. Several possibilities of extending the linear measurement range in microfluidic systems such as diffusion-limiting membranes and the flow injection setup are presented. Regarding the integration of enzymes into microfluidic systems during the fabrication process, the constraints imposed by the biomolecules due to the limited usage of high temperatures and solvents are addressed. In the second part, the most common forms of enzyme integration into microfluidic systems, i.e. in channels and on electrodes, on microparticles, on paper and thread and as injected enzyme solutions, are reviewed, focusing on fabrication, applications and performance.

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1. Introduction

Today, the field of electrochemical biosensors spans a very wide range, including bioreceptors such as nucleic acids, antibodies, aptamers, cells and enzymes (Grieshaber et al. 2008; Ronkainen et al., 2010). The electrical transducers coupled to the bioreceptors have evolved from Clark's oxygen electrode (Clark and Lyons, 1962) to sophisticated devices such as organic electrochemical transistors (Strakosas et al., 2015) and nanostructured electrodes (Karimi-Maleh et al., 2013, 2014). In this extensive research field, enzymatic sensors are among the most popular and most studied devices. This can be explained with the advantages of using enzymes for transduction: they offer high specificity towards their target molecules (Janata, 2009), can remain active for several weeks when kept in an appropriate environment, can be immobilized in various ways and are commercially available for a wide variety of substrates (Cass and Ligler, 1999; Moreno-Bondi and Benito-Peña, 2006). The possibility to measure important analytes like glucose, lactate or urea makes enzymes well suited for biosensors in the clinical field as well as for home use.

The advantages of enzymes for biosensing are particularly pertinent when combined with microfluidics. Microfluidic systems are employed to provide an integrated environment, where sample preparation, fluid control and measurement take place at a confined space with volumes in the nanoliter to milliliter range (Whitesides, 2006). They offer the possibility to perform measurements with a high degree of automation, independently from expensive and large laboratory equipment, using only small amounts of reagents and often providing faster reaction times and measurements (Hansen and Quake, 2003; Sackmann et al., 2014). The combination of enzymes and microfluidics has the potential to produce point-of-care (POC) assays for a broad range of analytes and applications.

When combining enzymes with microfluidics, several aspects have to be taken into account. One of them is enzyme integration into the system. This can either be done by immobilizing the biomolecules on a supporting surface such as electrodes, microfluidic channels, paper, thread or microparticles or by injecting them as a solution into the system at the time of measurement. These different methods are illustrated in Fig. 1. Associated with the method of integration is the choice of an appropriate immobilization procedure. A variety of methods ranging from simple adsorption to covalent linkage or bioaffinity bonding are available. All of them have their advantages and drawbacks dependent on the specific application.

The integration of enzyme immobilization into the fabrication process of the system is also an important issue since the sensitivity of enzymes to environmental conditions such as temperature (Minagawa et al., 2007; Zoldak et al., 2004) and chemical compounds (Stepankova et al., 2013) can restrict the feasibility of the methods of fabrication and assembly of the system.

The measurement performance of enzymatic sensors is affected by factors such as the Michaelis–Menten kinetics of enzymatic reactions (Ronkainen et al., 2010), the dependency of oxidase

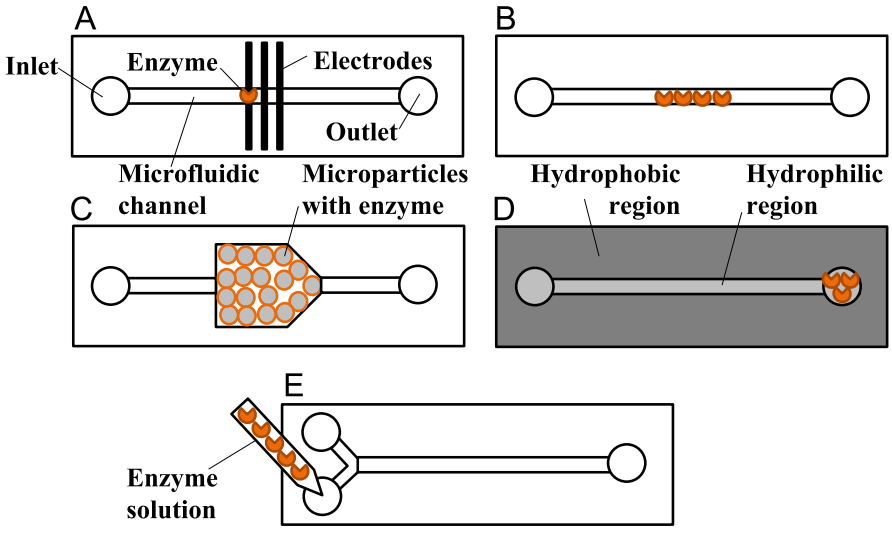

Fig. 1. Integration of enzymes into microfluidic systems: (A) on electrodes, (B) immobilized on channel walls, (C) on microparticles, (D) on paper with hydrophilic and hydrophobic regions and (E) introduction as a solution and mixing with an analyte.

enzymes on redox mediators and the limited linear measurement range associated with these factors (Banica, 2012). In microfluidic systems, these limitations can be obviated in several ways, and thus the measurement range extended.

This review intends to give an overview of enzymatic microfluidic biosensing systems categorized by their form of enzyme integration. In Section 2, typical immobilization methods, methods for the extension of the linear measurement range and detection techniques will be described. Also, the time of immobilization in the fabrication process will be discussed. In Sections 3–6, microfluidic systems with enzymes immobilized on electrodes, microfluidic channels, paper, thread and microparticles and those with injected enzyme solutions will be discussed with regard to fabrication, applications and performance. As the field of microfluidic enzymatic systems is extensive and the properties of a particular system depend on a variety of factors, each section presents an introductory overview of ongoing research.

2. Characteristics of enzymatic microfluidic systems

2.1. Immobilization

There exists a wide variety of immobilization methods for enzymes, ranging from simple adsorption on a support to more sophisticated approaches involving biotin–streptavidin bonding or entrapment in a polymer. Selection of the appropriate method depends on a number of factors, including immobilization surface properties, lifetime of the enzyme layer, complexity of the method and integration into the whole fabrication process. Fig. 2 and Table 1 present the most commonly used methods which will be described here. For a more detailed insight into the topic, the reader is referred to the review by Kim and Herr (2013).

2.1.1. Physisorption

One of the simplest immobilization methods predominantly used with electrodes is physical adsorption. The enzyme is dissolved in an aqueous solution (e.g. phosphate buffered saline) which is dispensed onto the support where the solvent evaporates. Intermolecular interactions such as electrostatic or van der Waals' forces then form the bond (Israelachvili, 2011). Often a crosslinker such as glutaraldehyde is added to the solution to covalently link pairs of enzyme molecules (see Subsection 2.1.2), thereby increasing their stability and avoiding detachment from the surface (Lopez-Gallego et al., 2005). Since no complicated protocols requiring several reagents have to be followed, physisorption offers the possibility to immobilize enzymes in a few minutes without further development steps (Kim and Herr, 2013). Disadvantages of this technique include potential detachment of enzymes due to weak bonds, poor control of orientation and molecule density and denaturation due to direct contact between enzyme and surface (Cass and Ligler, 1999). Despite these drawbacks, long-term stabilities of the enzyme layer up to 10 weeks with dry storage between measurements have been achieved with this technique (Ges and Baudenbacher, 2010b).

2.1.2. Covalent bonding

Covalent bonding is a more sophisticated technique which can be used on a variety of supports. It involves forming irreversible bonds between surface groups of the enzyme (typically NH_2 groups of the amino acid lysine) and surface groups of linker molecules bound to the support in a first step (Cass and Ligler, 1999). Linker molecules are available with a variety of functional head groups on both ends suited for different surfaces and immobilization protocols. While the first group matches the immobilization surface and forms a so-called self-assembled monolayer (SAM) on it, the second forms a covalent bond with the enzyme. Advantages of this approach are high uniformity of the layer, good control of the amount of immobilized enzyme, a smaller risk of denaturation and the possibility to incorporate antifouling agents like polyethylene glycol into the SAM (Banerjee et al., 2011; Frasconi et al., 2010). In contrast to adsorption, covalent bonding requires longer fabrication times, since the formation of the SAM and the subsequent linkage of the enzymes to it take several hours (Cass and Ligler, 1999). Also, care has to be taken to keep all chemicals as pure as possible so that homogeneity of the SAM is not compromised.

SAMs on gold or platinum electrodes can be formed with linker molecules containing a sulfhydryl (thiol) group such as mercaptopropionic acid or mercaptoethanol, the sulfur atom of which is able to form a strong bond with the metal atoms (Li et al., 2011; Y. Wang et al., 2010b). For the popular microfluidic materials polydimethylsiloxane (PDMS) and glass, compounds containing silane groups like APTES ([3-aminopropyl]triethoxysilane) are used (Sheng et al., 2012; Tan et al., 2008), as these are able to bind to the hydroxyl groups present on these surfaces. For other polymers such as poly(methylmethacrylate) (PMMA), substances like polyethylenimine can be used to form a SAM (Cerdeira Ferreira et al., 2013). Before functionalization, a pretreatment with acetone (Limbut et al., 2007) or NaOH (Cerdeira Ferreira et al., 2013) may be necessary to render the surface hydrophilic. Microparticles can be purchased with surface groups ready for enzyme immobilization (Kim and Herr, 2013). In the case of glass microparticles, silanization may be necessary (Kim et al., 2009).

Once the SAM has been formed, the enzyme is linked to the second head group of the linker molecules. Here, either carboxylic

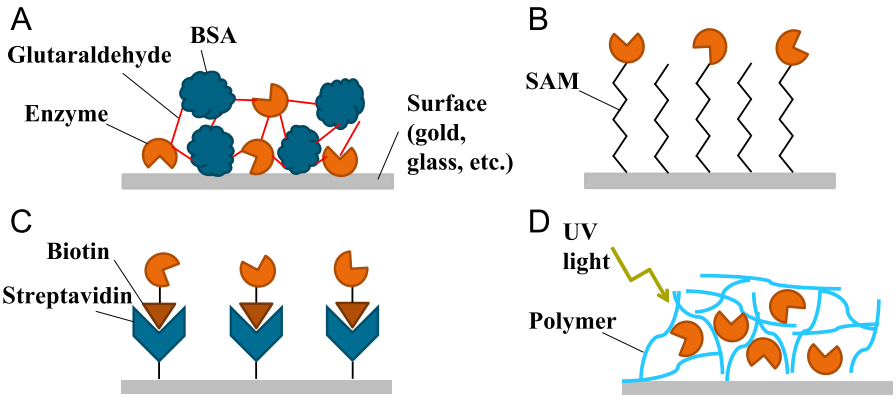

Fig. 2. Immobilization of enzymes: (A) cross-linking of adsorbed enzymes, (B) covalent bonding on self-assembled monolayer (SAM), (C) bioaffinity bonding and (D) entrapment in a polymer matrix.

Table 1
Typical immobilization methods in microfluidic enzymatic systems.

Immobilization method	Characteristics	Reported lifetime
Physisorption	Simple, takes only few minutes; Detachment and denaturation possible, poor control of molecule orientation	10 weeks (Ges and Baudenbacher, 2010b)
Covalent bonding	SAM with high uniformity, good control of enzyme density and orientation, reduced denaturation; Complex protocols, time-consuming	12 weeks (Srivastava et al., 2011)
Bioaffinity bonding	Good control of enzyme density and orientation, reduced denaturation, biotinylated enzymes available; Complex protocols, time-consuming	20% signal loss after 2 weeks (Anzai et al., 1998)
Entrapment in polymers	Enzymes in aqueous environment retain native form, little denaturation	18% signal loss after 1 week (Jang et al., 2012)

head groups with amide bonding or amine head groups for the formation of a Schiff base are typically employed. In the first case, the carboxylic group is usually activated with NHS (N-Hydroxysuccinimide) and EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) and then forms an amide bond with the NH_2 group of the enzyme (Srivastava et al., 2011; Y. Wang et al., 2010b). In the second case, glutaraldehyde is bound to the amine head group of the linker molecule by forming a Schiff base with one aldehyde group. The enzyme can then be bound to the second aldehyde group *via* surface NH_2 groups, also forming a Schiff base (Sheng et al., 2012).

For microfluidic systems with covalently bonded enzymes, long-term stabilities up to 12 weeks have been reported (Srivastava et al., 2011) which is two weeks longer than the stability of adsorbed and cross-linked enzyme layers (Ges and Baudenbacher, 2010b). However, the longer and more complicated immobilization procedure has to be taken into account.

2.1.3. Bioaffinity bonding

Bioaffinity bonding is based on the strong affinity of certain pairings of biomolecules to each other. The most popular example is the binding of biotin, a B-vitamin, to the protein streptavidin, or the similar protein avidin. The tetrameric protein streptavidin has a strong affinity for the vitamin biotin with a dissociation constant of about 10^{-14} mol/L which is one of the strongest naturally occurring non-covalent bonds (Green, 1975). To make use of this reaction, biotin has to be covalently bound to the enzyme while streptavidin is bound to the support. When the biotinylated enzyme is brought into contact with the support, a strong bond forms spontaneously (Kim and Herr, 2013). Similar to covalent bonding, advantages include good control of enzyme density, reduced denaturation and better control on molecule orientation than physisorption. Fornera et al. employed this technique in microfluidic channels by first forming a dendronized polymer layer on PDMS (Fornera et al., 2012). Avidin was then covalently bound to the polymer, making the channels ready for immobilization of biotinylated glucose oxidase and horseradish peroxidase. If the immobilization of biotin and streptavidin is supposed to be avoided, microparticles with streptavidin on their surface as well as biotinylated enzymes are commercially available, making bioaffinity bonding a convenient immobilization method for this support (Kim and Herr, 2013). However, the higher cost of such ready-made products has to be considered.

2.1.4. Entrapment in polymers

In contrast to the techniques described so far, entrapped enzymes are not in direct contact with the solution to be measured. The biomolecules are embedded into a three dimensional polymer matrix which is permeable to aqueous solutions and able to incorporate a significant amount of water (Jang et al., 2012). Entrapment in such hydrogels is considered a very gentle immobilization method since biomolecules are kept in their native form while being protected from dehydration (Ikeda et al., 2010).

Electropolymerization is a well suited technique for entrapping enzymes in hydrogels if electrodes are present in the microfluidic system. Monomer solutions like 3,4-ethylenedioxythiophene (Nien et al., 2011), aniline and o-phenylenediamine (Cheng et al., 2006; Huang et al., 2011) or pyrrol (Blanco-Gomez et al., 2009; Huang et al., 2007) are mixed with an enzyme solution and brought into contact with the electrodes. By applying a constant current or a cyclic voltage sweep between the working and counter electrode for a few minutes, the monomer polymerizes on the electrode and entraps the enzyme.

For the functionalization of microchannels, polymerization of mixtures of poly(vinyl alcohol) (PVA) or poly(ethylene glycol) diacrylate (PEG-DA) and an enzyme by irradiation with ultra-violet (UV) light is common (Jang et al., 2012; Koh and Pishko, 2005; Suzuki and Matsugi, 2005). By polymerizing solutions of PEG-DA and enzymes, also hydrogel microparticles can be formed. The mixture is therefore irradiated through a photomask, producing microparticles outside the system or directly in the microfluidic channels or chambers (Jang et al., 2012; Kim et al., 2009).

2.2. Integration aspects

Besides selecting the appropriate bonding chemistry, the point in time of enzyme integration in the fabrication process is another important choice to make. Enzymes can be integrated at two points of the process:

- before or
- after assembly of the system.

When functionalized before assembly, the support is directly accessible and can thus be functionalized using simple techniques like droplet dispensing or immersion into an enzyme solution. Advantages of this approach are low fabrication costs and the possibility of batch fabrication. The integrated enzyme, however, imposes constraints on the following fabrication steps: if a specific temperature is exceeded, enzymes are irreversibly damaged and lose their activity. For the frequently used enzymes glucose oxidase and lactate oxidase, this is the case at 56 °C (Zoldak et al., 2004) and 70 °C (Minagawa et al., 2007), respectively. Additionally, solvents and other chemicals required for the fabrication of the microfluidics can damage the biomolecules (Stepankova et al., 2013).

These constraints greatly reduce the choice of bonding methods. When the microfluidics are made from the polymer PDMS, often simple and reversible mechanical clamping of the microfluidic layers is used, possibly combined with a plasma pretreatment to enhance adhesion. Whereas this method proves to be efficient in a lab environment where simple and reversible sealing is preferable, it may not be the best choice for commercial systems since the reliability of oxygen plasma treatments can vary greatly (Duffy et al., 1998) and clamped structures can be broken by the user unintentionally. A possible alternative for sealing the

microfluidic layers at room temperature after functionalization is the usage of UV curable glue (Ahn et al., 2004; Do et al., 2008). Contact between glue and enzymes should however be avoided.

If the support is functionalized after assembly of the microfluidic system or the enzymes introduced as a solution at the time of measurement, there are no restrictions regarding the fabrication process since there are no biocomponents sensitive to temperature and chemicals in the system at this point. Hence, a strong and reliable bond between the microfluidic layers can be achieved by thermal bonding (Cerdeira Ferreira et al., 2013). On the other hand, for functionalization the enzyme solution has to be introduced into the system through a channel, since there is no direct access to the inner parts anymore (Y. Wang et al., 2010b). As a consequence, the immobilization procedure and precise control of the immobilization location become more complicated and higher amounts of chemicals have to be used. There is also the issue of unintended adsorption of biomolecules to the channel walls during functionalization, making extensive rinsing necessary.

2.3. Extension of the linear measurement range

The kinetics of enzymatic reactions are governed by the Michaelis–Menten model which relates the reaction rate v to the substrate concentration s (Banica, 2012):

$$v = v_{max}\, s/(K_m + s). \quad (1)$$

K_m is the Michaelis constant which quantifies the affinity of the enzyme for its substrate and v_{max} is the maximum reaction rate. Since several factors such as temperature, pH, immobilization technique and diffusion-limiting membranes influence these numbers (Janata, 2009), they have to be determined for the specific application. The linear measurement range of enzymatic sensors is limited to substrate concentrations well below K_m. In this range, the reaction rate is diffusion limited and grows nearly linearly with an increase in substrate concentration up to half of its maximum value (Banica, 2012); this is depicted in Fig. 3. For higher concentrations, the reaction rate shows hyperbolic behavior before reaching its maximum v_{max}, consequently, the measurement signal (current, fluorescence, color change) derived from the enzymatic reaction is also not a linear measure for the substrate concentration any longer.

In addition, the reactions of oxidases, such as glucose oxidase (GOx) or lactate oxidase, require an oxidizing agent or "redox mediator" such as oxygen which accepts electrons from the enzyme molecules after the first step of the reaction (Eqs. (2) and (3)) (Ronkainen et al., 2010):

$$\beta - D - \text{Glucose} + \text{GOx} - \text{FAD} \rightarrow \text{GOx} - \text{FADH}_2 + \delta - D - \text{gluconolactone} \quad (2)$$

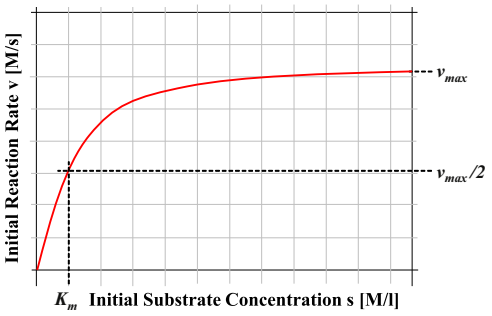

Fig. 3. Michaelis–Menten kinetics of enzyme reactions; up to K_m the relationship between substrate concentration s and reaction rate v can be considered linear.

$$\text{GOx} - \text{FADH}_2 + \text{O}_2 \rightarrow \text{GOx} - \text{FAD} + \text{H}_2\text{O}_2 \quad (3)$$

$$\text{H}_2\text{O}_2 \rightarrow 2e^- + \text{O}_2 + 2\text{H}^+ \quad (4)$$

The redox mediator converts the enzyme back into its native form, making it ready for conversion of the next substrate molecule. In amperometric detection, the reduced oxygen molecule is oxidized at the working electrode and the generated current measured (Eq. (4)) (Wang, 2006). If there is a lack of oxygen in the sample solution, the enzymes are not immediately converted back to their initial form and thus are not directly ready for digestion of the next substrate molecule. The reaction rate then becomes limited by the oxygen supply and the linear range is lower than in solutions saturated with oxygen (Harrison et al., 1988). Several measures to overcome these limitations can be taken as described in the following subsections, depicted in Fig. 4 and listed in Table 2.

2.3.1. Diffusion-limiting membranes

Diffusion-limiting membranes limit the flux of substrate molecules to the enzyme layer while not impeding oxygen supply (Harrison et al., 1988). The linear range is extended, however at the expense of a smaller measurement signal and a longer response time for the same concentration. Besides their diffusion-limiting behavior, membrane materials should be nontoxic, easy to process and highly permeable to oxygen. Frequently used materials that fulfill these requirements are Nafion (Ges and Baudenbacher, 2010a,b; Li et al., 2013; Rodrigues et al., 2008; Yi et al., 2012), polyurethane (Ahn et al., 2004; Li et al., 2008; Trzebinski et al., 2012) and cellulose (Bindra et al., 1991; Vaidya and Wilkins, 1994). Membranes are typically used with electrodes on which they can be formed by dispensing, spin coating or dipping into an aqueous solution of the membrane material and letting the solvent evaporate. A comparative study of enzymatic glucose sensors with and without Nafion membranes demonstrated an extension of the linear measurement range from 2.5 mM to 30 mM (Harrison et al., 1988). With polyurethane membranes, linear ranges up to 40 mM for glucose have been demonstrated (Koudelka et al., 1989).

2.3.2. Redox mediators

Instead of relying on oxygen as the oxidizing agent in the enzymatic reaction, other substances can be used. These redox mediators are reduced in the second part of the enzymatic reaction and can easily be oxidized on the working electrode, thereby transporting electrons from the enzyme to the electrode (Ronkainen et al., 2010). Different from oxygen, they can be co-immobilized with the enzyme or added to the sample solution in excess so that shortages are avoided and the linear range extended. Picher et al. examined the redox mediators hydroquinone, ferricyanide, 8-hydroxyquinoline, anthraquinone and menadione for their compatibility with glucose oxidase and signal strength of the amperometric read out circuit (Picher et al., 2013). Ferricyanide was found to have the best biocompatibility and give the strongest measurement signal.

2.3.3. Flow injection

As an alternative to modifying the sensor structure, the measurement range can be extended by lowering the analyte concentration in the sample solution. This can be done by using flow injection analysis (FIA) (Backer et al., 2013; Hernandez et al., 2013; Ito et al., 2011; Y. Wang et al., 2010b; Yi et al., 2012). It is based on injecting a defined amount of the solution to be measured into a stream of a buffer solution flowing through the microfluidic system (Ranger, 1981). Due to the laminar flow conditions in microfluidic channels and diffusion of analyte into the buffer, the injected sample disperses into a wide band with a lower

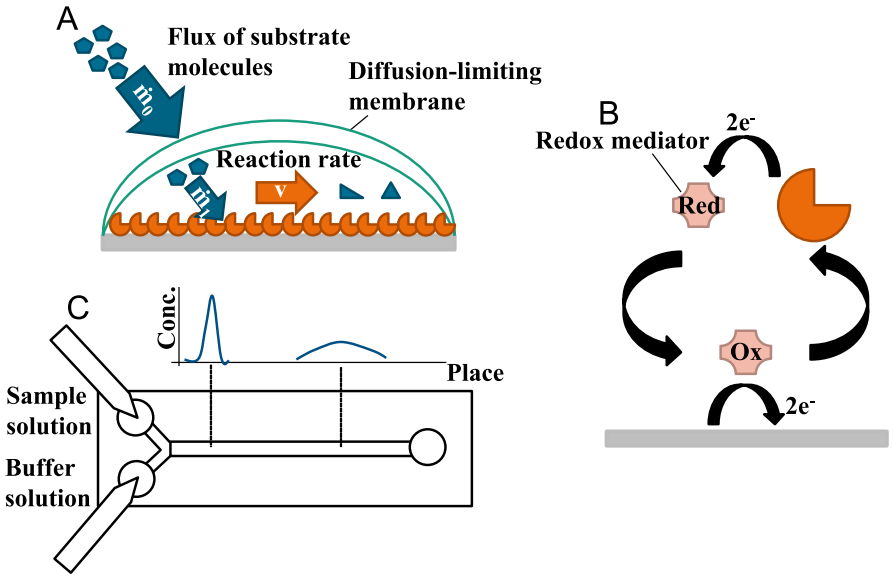

Fig. 4. Extension of the linear measurement range: (A) diffusion-limiting membranes, (B) redox mediators and (C) flow injection using dispersion of the analyte.

Table 2
Typical methods for extension of the linear measurement range in microfluidic enzymatic systems.

Method of extension	Characteristics	Reported linear range
Diffusion-limiting membranes	Flux of substrate molecules is limited; signal decrease, longer response time	Glucose: 40 mM (Koudelka et al., 1989)
Redox mediators	Transports electrons from enzyme to electrode, avoids shortages in oxygen supply for oxidases	Depends on sensor structure; see for example (Cass et al., 1984)
Flow injection	Analyte concentration is lowered by injection into buffer stream; requires external equipment	Glucose: 20 mM (Backer et al., 2013)

concentration which is measured in the system. As external pumps are needed for this type of measurement, it is more suited for a laboratory environment than for POC use.

2.4. Detection principles

The majority of systems discussed in this review employs only a few detection techniques which will be described in the following. These are schematically depicted in Fig. 5 and summarized in Table 3. Wider and more detailed insight into measurement techniques for enzymatic sensors can be found in work dedicated to electrochemical measurements and optical detection techniques (Baldini et al., 2006; Grieshaber et al., 2008).

2.4.1. Amperometry

Amperometry is the predominant technique for systems

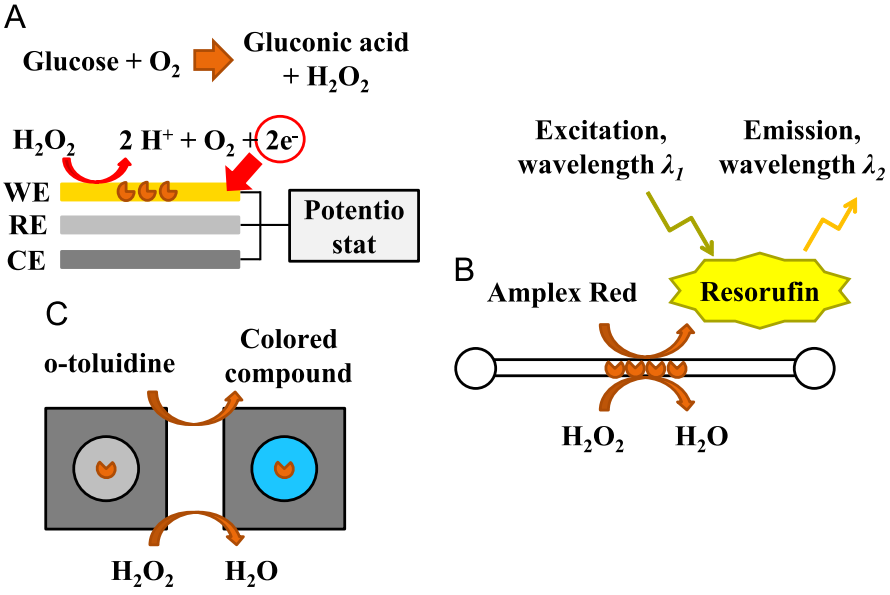

Fig. 5. Common detection principles in microfluidic enzymatic systems with exemplary applications: (A) amperometric detection of glucose, (B) fluorescence detection of H_2O_2 using Amplex Red® and (C) colorimetric detection of H_2O_2 using o-toluidine.

Table 3
Typical detection principles in microfluidic enzymatic systems.

Detection principle	Characteristics	Reported dectection limit
Amperometry	Straightforward detection scheme, affordable electronic equipment; requires integrated electrodes, stability issues with reference electrode	Glucose: 2.5 μM (Cerdeira Ferreira et al., 2013)
Fluorescence and absorbance measurement	Low detection limits, no integration of electrodes; addition of reagents such as Amplex Red® required	Glucose: 200 nM (Nakajima et al., 2006)
Colorimetry	Often used with paper-based systems, readout by eye possible; color intensity on paper depends on several factors, color development can take up to 30 minutes	Glucose: 23.8 μM (Cocovi-Solberg et al., 2012); Glucose by eye: 0.5 mM (Dungchai et al., 2010)

containing electrodes. The popularity of this method can be largely attributed to the straightforward detection scheme (i.e. oxidation of H_2O_2 when using oxidases) and the widespread availability of dedicated and affordable laboratory instruments. Detection limits typically are in the low μM range, however measurements down to a few pM are possible with microelectrodes (Hayashi et al., 2005). Amperometry requires a three electrode setup in which the functionalized working electrode (WE) is held at a defined potential relative to a reference electrode (RE) which typically is an Ag/AgCl electrode (Ronkainen et al., 2010). The product of the enzymatic reaction (e.g. H_2O_2) is oxidized at the WE and the released electrons generate a current detected by a potentiostat connected to the WE. A third counter electrode (CE) provides the current needed to keep the WE at the desired potential. Stability of the Ag/AgCl electrode can be a critical issue and is subject to ongoing research (Huang et al., 2009), which is why in some systems a replaceable external reference electrode is used (Ges and Baudenbacher, 2010a; Srivastava et al., 2011). This, however, adds an additional part to the system which has to be integrated. Since the measurement signal is transferred electrically, the material of the microfluidics does not have to be transparent as for optical measurement techniques. On the other hand, conducting paths are required to connect electrodes and read out electronics.

2.4.2. Fluorescence and absorbance measurement

Fluorescence and absorbance measurements do not require electrodes integrated into the microfluidic system and offer low detection limits in the nanomolar range (Nakajima et al., 2006; Tan et al., 2008). In order to yield a fluorescence signal or a color change, reagents such as Amplex® Red or DTNB (5,5-dithio-bis-2-nitrobenzoic acid) have to be added to the sample solution. They react with the product of the enzymatic reaction and form fluorescent products such as resorufin (Brotea and Thibert, 1988) or colored compounds (Tan et al., 2008). Irradiation of these products and measurement of the emission or absorbance can then be carried out by a fluorescence microscope or a spectrometer. Also, the microfluidics have to be transparent to the wavelength of the light used in the detection scheme, at least at the measurement location. Interesting approaches to eliminate the additional reagent by incorporating quantum dots (Jang et al., 2012) or pH sensitive fluorophores (Koh and Pishko, 2005; Russell et al., 1999) together with enzymes in hydrogels have been reported.

2.4.3. Colorimetry

Colorimetry is often used for paper-based systems. It transfers the reaction of the enzyme with its substrate into a visible color change (Free et al., 1960). Indicators like o-toluidine are co-immobilized with the enzymes and react with the product of the enzymatic reaction, forming a colored compound. Development of the full color intensity takes from several minutes up to 30 min (Chen et al., 2012). The intensity of the color is in a certain range proportional to the substrate concentration but also depends on factors such as enzyme concentration, drying conditions of enzyme solution, pH value of the sample solution and color of the paper substrate (Chen et al., 2012). It can be recorded using for example a camera or an office scanner (Abe et al., 2008; de Souza et al., 2012; Maattanen et al., 2011) and converted into numerical values using image analysis software. If the measurement is intended to be carried out without any electronics, the color can be determined by eye (Dungchai et al., 2010; Lu et al., 2009) and matched with a reference color table.

2.5. Applications

The majority of the proposed systems targets the biomedical sector, either as small sized point-of-care assays for clinical and home use or as more complex systems coupled with flow injection analysis and optical readout. Due to the prevalence of diabetes, the by far most frequently detected analyte in this sector is glucose (Heller and Feldman, 2008). Since glucose oxidase is a comparatively cheap and broadly available enzyme, glucose is also often used as a model analyte in enzymatic biosensing. Other analytes in the biomedical sector include lactate (Kurita et al., 2006; C.Y. Li et al., 2010a), cholesterol (Aravamudhan et al., 2007; Wisitsoraat et al., 2010) and renal markers such as urea (Chang et al., 2011; Malecha et al., 2009), creatinine (Satoh et al., 2008; Suzuki and Matsugi, 2005), alanine aminotransferase (Song et al., 2009) or glutamic-oxaloacetic transaminase (Ohgami et al., 2007). In food and environmental monitoring, enzymatic biosensing systems have been developed for the detection of fructose (Atalay et al., 2012), lactose in milk (Fornera et al., 2012; Ito et al., 2011), pesticide residues in vegetable and soil samples (Duford et al., 2013) or hypoxanthine to determine the freshness of fish meat (Dolmaci et al., 2012; Lawal and Adeloju, 2010). Another interesting application field is the monitoring of nutrients and metabolic products in cell cultures and biotechnological processes. In this context, important analytes are glucose (Ges and Baudenbacher, 2010a; Rodrigues et al., 2008), lactate (Cheng et al., 2006), but also glutamine and glutamate (Backer et al., 2013).

3. Enzymes in microfluidic channels and on electrodes

3.1. Fabrication

An inherent feature of virtually all microfluidics lending itself for functionalization is microfluidic channels. These can be produced in various ways and materials. One of the most popular choices is softlithographic patterning of polydimethylsiloxane (PDMS), an organic polymer based on silicon. The material is poured into a mold which defines the microfluidic structures, cured and finally detached from the mold. Other polymers like poly(methyl methacrylate) (PMMA) or polystyrene can be processed by techniques such as laser micromachining (Cerdeira Ferreira et al., 2013; Tan et al., 2008) or hot embossing (Ellis et al., 2008). Channels can also be defined by cutting adhesive tape on a glass slide into a desired shape (Tangutooru et al., 2012). The formed part is then sealed with another part of the same or a different material, potentially leading to channel walls with different properties. If no sophisticated microfluidic features are

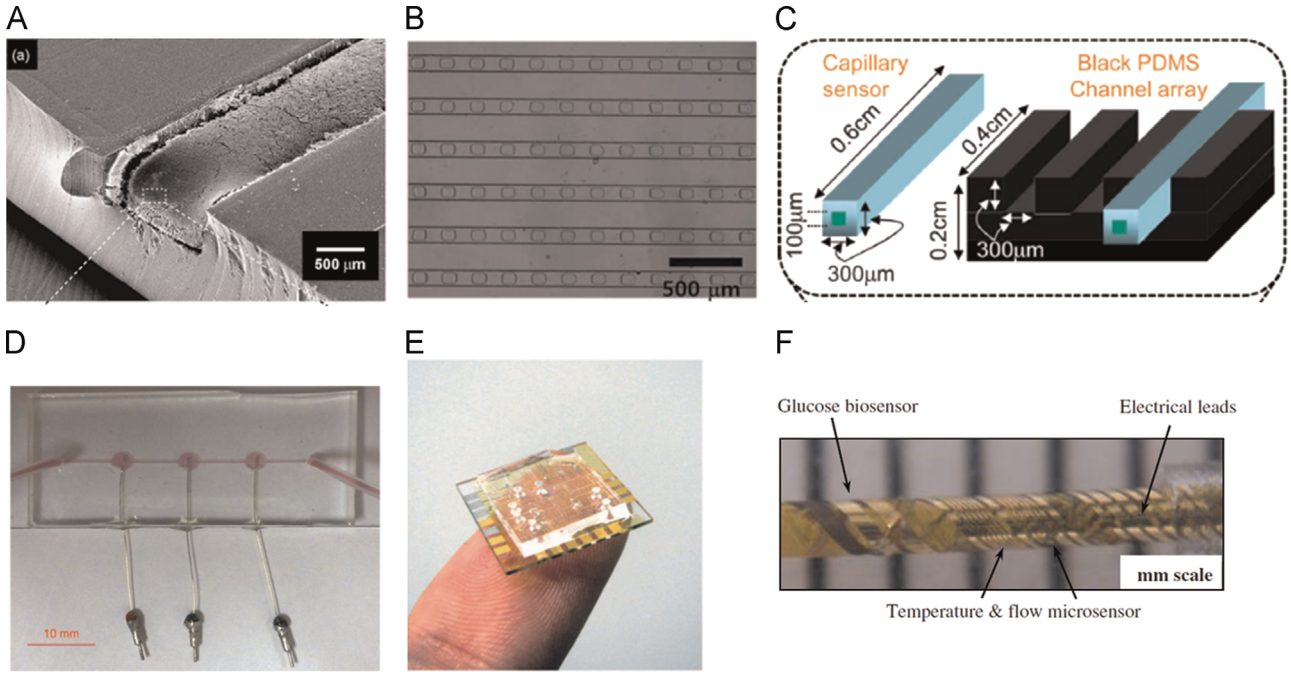

Fig. 6. Enzymes immobilized (A) in microfluidic channel, adapted from Morimoto et al. (2007), Copyright 2007, with permission from Elsevier, (B) in hydrogel particles, adapted from Jang et al. (2012), Copyright 2012, with permission from Elsevier, (C) in glass capillaries, adapted from Kimura et al. (2012) with permission of The Royal Society of Chemistry, (D) on wire electrodes, adapted from Lamberti et al. (2012) with permission from AIP Publishing LLC, (E) on planar electrodes, adapted from Satoh et al. (2008) and (F) on flexible electrodes, adapted from Li et al. (2008), Copyright 2008, with kind permission from Springer Science and Business Media.

required, glass capillaries are a simple alternative to channel networks produced in polymers or glass (Fig. 6C) (Inadumi et al., 2007; Kimura et al., 2012).

One of the most typical structures integrated into microfluidics and used for transduction are electrodes. For enzymatic sensing applications, the noble metals gold and platinum are the most common materials as these are inert, highly conductive and form stable films on many supporting surfaces. To produce electrodes on a flat surface (Fig. 6E), evaporation or sputtering of gold or platinum in combination with photolithographic steps, etching or lift-off processes is required. Besides the commonly used but fragile substrate glass, electrodes can be produced on polymer materials including polycarbonate (Y. Wang et al., 2010b), cyclic olefin copolymer (COC) (Ahn et al., 2004) and poly(methyl methacrylate) (PMMA) (Kurita et al., 2006), which are non-breakable and can be processed by mass fabrication methods like injection molding. Electrodes on flexible substrates such as Kapton film allow realization of lab-on-tube systems (Fig. 6F) (Li et al., 2008; C.Y. Li et al., 2010a) while microspike electrodes can be suited for application as transdermal sensors (Trzebinski et al., 2012). Commercially available wire electrodes can be a straightforward alternative to complicated fabrication procedures but leak-proof integration requires additional steps such as sealing with UV curable glue (Fig. 6D) (Lamberti et al., 2012).

Carbon nanotubes, metal nanowires and metal nanoparticles are the smallest types of electrodes which have been functionalized with enzymes and integrated into microfluidics (Aravamudhan et al., 2007; C.Y. Li et al., 2010a; Li et al., 2013; Wisitsoraat et al., 2010; Yu et al., 2013). As these, however, have to be fixed to a supporting electrode, the dimensions of the resulting sensor still remain at least in the micrometer range. The integration of nanostructures into a microfluidic system can involve growth of CNTs on a supporting layer (Wisitsoraat et al., 2010), chemical bonding of nanowires to the support (Yu et al., 2013), physical adsorption (Li et al., 2013) or alignment of nanowires between two supporting electrodes through dielectrophoresis (Aravamudhan et al., 2007).

3.2. Functionalization

Enzymes can be immobilized in microfluidic channels in two ways: on an entire part of the microfluidic device or at specific locations. In the first case, a part of the system, i.e. a channel or chamber, is incubated completely with enzyme solution (Fig. 6A) (Cerdeira Ferreira et al., 2013; Picher et al., 2013; Limbut et al., 2007; Morimoto et al., 2007). This approach is well suited for first experiments since neither a sophisticated microfluidic design nor complicated fabrication steps are necessary. To increase the surface of the functionalized part and thereby the accessible enzymes, structures like pillars can be incorporated into the microfluidic design (Hayashi et al., 2005). In the course of functionalization, also an antifouling coating can be applied to the microfluidics (Picher et al., 2013).

On the other hand, the functionalization of microfluidic channels or chambers at specific locations requires more complex designs and methods. Channels can be filled with mixtures of photoreactive compounds like poly(ethylene glycol) diacrylate (PEGDA) and an enzyme and then irradiated with UV light through a photomask, forming discrete hydrogel spots incorporating the enzyme (Fig. 6B) (Jang et al., 2012; Koh and Pishko, 2005). The microfluidic system can be divided by valves into different compartments which are functionalized separately (Fornera et al., 2012). A more unusual method is the laser-induced forward transfer (LIFT) technique which transfers enzyme molecules from a glass slide onto specific locations on a substrate through laser irradiation (Tsuboi et al., 2007).

For electrodes, the different bonding chemistries of gold or platinum and microfluidic materials such as PDMS or glass allow selective functionalization through covalent bonding employing linker molecules with a thiol group (see Subsection 2.1.2.). After functionalization, a rinsing step is advisable to remove enzymes nonspecifically adsorbed to the channel walls (Y. Wang et al., 2010b). If immobilization is performed before assembly of the system, simple adsorption followed by crosslinking of the enzymes is also common.

3.3. Characteristics and performance

A main advantage of functionalizing electrodes or channels at specific locations is the precise control of the measurement location inside the microfluidic system. Precise functionalization is a prerequisite for the detection of multiple substances in parallel (Satoh et al., 2008), designs involving consecutive enzymatic reactions (Fornera et al., 2012) and measurements in very small volumes down to the picoliter range for the detection of metabolites secreted by single cells (Cheng et al., 2006; Ges and Baudenbacher, 2010a,b). Especially electrodes are often employed for parallel detection of multiple analytes, since individual readout of the signals can easily be achieved (Satoh et al., 2008). Also, the signals of different measurement sites can be applied against each other to calculate the difference in concentration at two points of the same system (Rodrigues et al., 2008). However, the more sensors are integrated, the more difficult selective functionalization of the electrodes becomes. Furthermore, cross interference between different enzymatic sensors is a major issue related to multi-analyte sensing. This is especially problematic when enzymes of the same kind, e.g. glucose oxidase, lactate oxidase or glutamate oxidase are used, since all of these use hydrogen peroxide oxidized on the electrodes.

Different approaches exist for individual functionalization and decoupling of sensors integrated into the same system. A common method is the usage of multiple channels for the different sensors (Fig. 7A) (Huang et al., 2011; Satoh et al., 2008; Yi et al., 2012). In this case, equal splitting of the stream of solution is of great importance since the flow rate can have significant influence on the measurement (Hashimoto et al., 2006; Lamberti et al., 2012). Alternatively, multiple electrodes can be placed in the same channel (Frey et al., 2010; Parra-Cabrera et al., 2012). In order to avoid cross-talk, the electrodes can be placed parallel to each other along the direction of flow and functionalized individually (Fig. 7C). Since flow in microfluidic channels is laminar (Whitesides, 2006), products of the enzymatic reactions are transported away from the electrodes without influencing the other measurements. In order to selectively functionalize the electrodes, systems with a dedicated functionalization channel for each electrode (Frey et al., 2010) and those utilizing laminar co-flow have been proposed (Parra-Cabrera et al., 2012). While these approaches obviate the problem of different flow rates in the channels, they complicate the microfluidic design.

Backer et al. (2013) proposed a system for the simultaneous measurement of glutamine, glutamate and glucose with electrodes lying one after the other in the same microfluidic channel. By incorporating catalase membranes in between the electrodes, the hydrogen peroxide produced by one sensor was decomposed to water and oxygen so that it did not influence the sensors located further downstream (Fig. 7B). Controlled placement of the

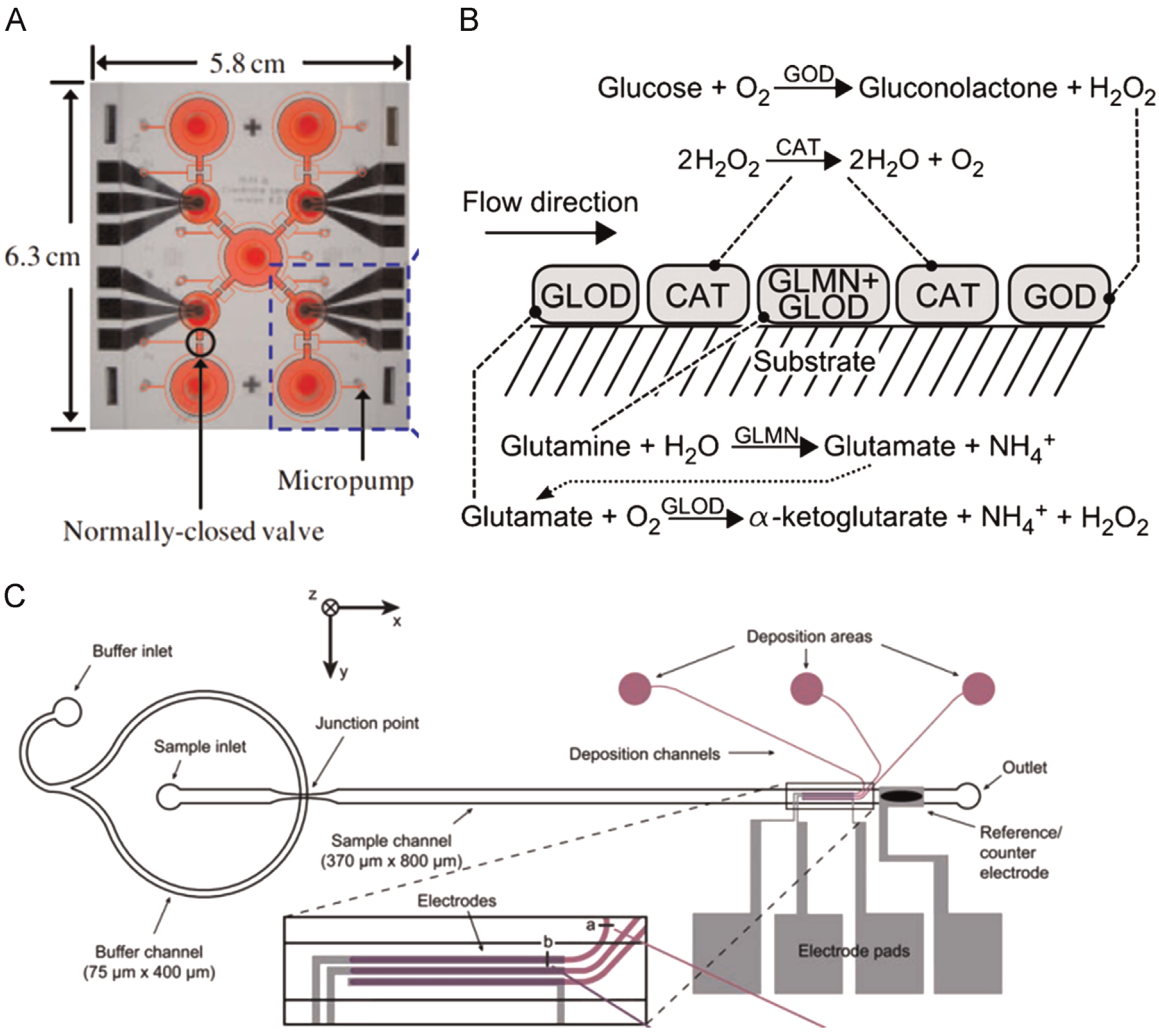

Fig. 7. Parallel sensing with electrodes: (A) multiple channels, adapted from Huang et al. (2011), Copyright 2011, with permission from Wiley, (B) decoupling with catalase membranes (Backer et al., 2013), Copyright 2013, reprinted with permission from Elsevier and (C) electrodes lying parallel along the direction of flow, adapted from Frey et al. (2010) with permission of The Royal Society of Chemistry.

different enzyme membranes however required direct access to the microfluidic channel.

The detection limit for systems with electrodes typically extends to the low μM range for glucose (Y. Wang et al., 2010b) and lactate (Cheng et al., 2006; Y. Wang et al., 2010b), while linear measurement ranges up to around 30 mM for these analytes are common when diffusion-limiting membranes are employed (C.Y. Li et al., 2010a; Trzebinski et al., 2012). The combination of enzymes immobilized on channel walls and optical detection can measure to the nM range (Nakajima et al., 2006; Tan et al., 2008); however, linear ranges are often limited to around 10 mM (Cerdeira Ferreira et al., 2013; Fornera et al., 2012).

4. Enzymes on micro and nanoparticles

4.1. Fabrication

In the systems described in the last section, enzymes were immobilized on parts which are permanently incorporated into the system, i.e. electrodes or channels. As an alternative, enzymes can be immobilized on micro or nanoparticles. These are commercially available in a variety of shapes (spheres, beads, bars), sizes (in the nm and μm range) and materials (polymers, agarose, gold, glass) and can be purchased with surface groups like NH_2 ready for functionalization (Kim and Herr, 2013). As an alternative, enzymes can be incorporated into hydrogel microparticles produced *in situ* from precursor solutions like PEG-DA (Jang and Koh, 2010; Kim et al., 2009) which are mixed with the enzyme solution and polymerized using UV irradiation.

When using microparticles in a microfluidic system, a method to retain the particles in the system has to be devised. One possibility is the definition of microfluidic structures including steps (Ito et al., 2011; Malecha et al., 2009) or micropillars (Jang and Koh, 2010; Kim et al., 2009) which stop the movement of the particles in the fluid stream at one point of the system. Wires incorporated into microfluidic channels can serve the same purpose (Blanes et al., 2007). The other typical method to retain microparticles is the usage of magnetic particles (Hernandez et al., 2013; Lin et al., 2013; Sheng et al., 2012). These can be kept at a desired position using an external magnetic field, making additional features in the channels unnecessary. When the magnet is removed or the magnetic field blocked, the particles are flushed out of the system.

Instead of physically retaining the particles in the system, these can also be incorporated into the material of the microfluidics. For PDMS, incorporation of silica particles into the material during the molding process (Zhang et al., 2006) and *in situ* generation of gold nanoparticles on the surface of the channel walls (Zhang et al., 2008) are two possible ways. External functionalization and replacement of the particles is then not possible, however.

An issue that has to be taken into account when using microparticles is their flow resistance. When particles with a diameter below 10 μm are used, a pressure of a few tens of kilopascals can be necessary to pump fluids through the system (Wang et al., 2008). For systems containing pressure sensitive parts, monitoring the pressure with an external gauge might be advisable.

4.2. Functionalization

A distinct advantage of microparticles is the possibility to functionalize the particles in a beaker outside the system. It simplifies the immobilization procedure and allows preparation of bigger amounts of microparticles which can then be split up and used in multiple systems. Since immobilization takes place outside the system, also the fabrication process of the microfluidics is not limited by the biomolecules, making usage of a broader variety of solvents and high temperatures possible. Malecha et al. made a microfluidic system from multiple ceramic layers which were laminated onto each other and fired at 875 °C – the high temperature could be used because enzymes were immobilized on microparticles and introduced into the system after assembly (Malecha et al., 2009).

A second advantage is the fact that microparticles can be easily introduced and removed from microfluidic systems, making easy renewal of the enzyme layer possible. Since enzymes lose their catalytic activity over time, the enzyme layer has to be periodically renewed or after a certain number of measurements, alternatively the system has to be disposed. If the biomolecules are immobilized on stationary features of the microfluidic system, regeneration involves multiple steps including washing and new functionalization (Limbut et al., 2007; Luo et al., 2008). Microparticles can be replaced in two simple steps by flushing the old ones out of the system and injecting new ones.

4.3. Characteristics and performance

A feature of microparticles that has been employed in several systems is that the enzyme loading in the system can be controlled in various ways. Ito et al. suggested reducing the size of the microparticles to increase the surface-to-volume ratio and thus the enzyme loading which could enhance the sensitivity of the measurement (Ito et al., 2007). Other possibilities include changing the dilution of the particle suspension (Godino et al., 2010), using magnetic particles and changing the length of the external holding magnet (Sheng et al., 2012) or incorporating microparticles into PDMS microfluidic channels of varying lengths (Zhang et al., 2006). For the latter two approaches, an approximately linear relationship between the amount of particles and limit of detection and linear range could be demonstrated.

Using microparticles for detection of multiple analytes or multi-step reactions requires additional effort since the signals of particles for different analytes have to be distinguished. For two-step reactions, microparticles can be functionalized with different enzymes and introduced into separate channels connected by capillary tubing afterwards. The conversion of lactose in milk samples to glucose in the first column and hydrogen peroxide in the second column is an example for this technique (Ito et al., 2011). The connection of the tubing, however, bears the risk of leakages. To circumvent this problem, microparticles can be generated inside separate microfluidic chambers *via* UV irradiation of a precursor solution through a photo mask (Fig. 8A) (Kim et al., 2009). Chambers with different particles then have to be separated by dedicated structures to avoid mixing of the particles.

Shape and color of microparticles are two properties which can be varied to measure multiple substances in the same system (Choi et al., 2008; Jang and Koh, 2010; Zhu and Trau, 2012). Jang and Koh produced particles containing a fluorescent dye and either glucose oxidase or alcohol oxidase through UV irradiation. Glucose oxidase particles were produced in round shape whereas alcohol oxidase particles had square shape (Fig. 8C). Thus, the fluorescence signals of the particles could be distinguished from each other when sample solutions containing both analytes were tested (Jang and Koh, 2010). The incorporation of fluorescent dyes with different colors in hydrogel microparticles is a related method for the distinction of measurement signals using optical detection (Fig. 8B) (Choi et al., 2008). Automated evaluation of the fluorescence image would however require algorithms to determine shape or color of the particles.

Besides being a support for enzyme immobilization, microparticles can also serve as “nanovalves”. Hydrogel nanoparticles made from poly(N-isopropylacrylamide) (PNIPAM) and poly(methacrylic acid) (PMAA) were immobilized with glucose oxidase in

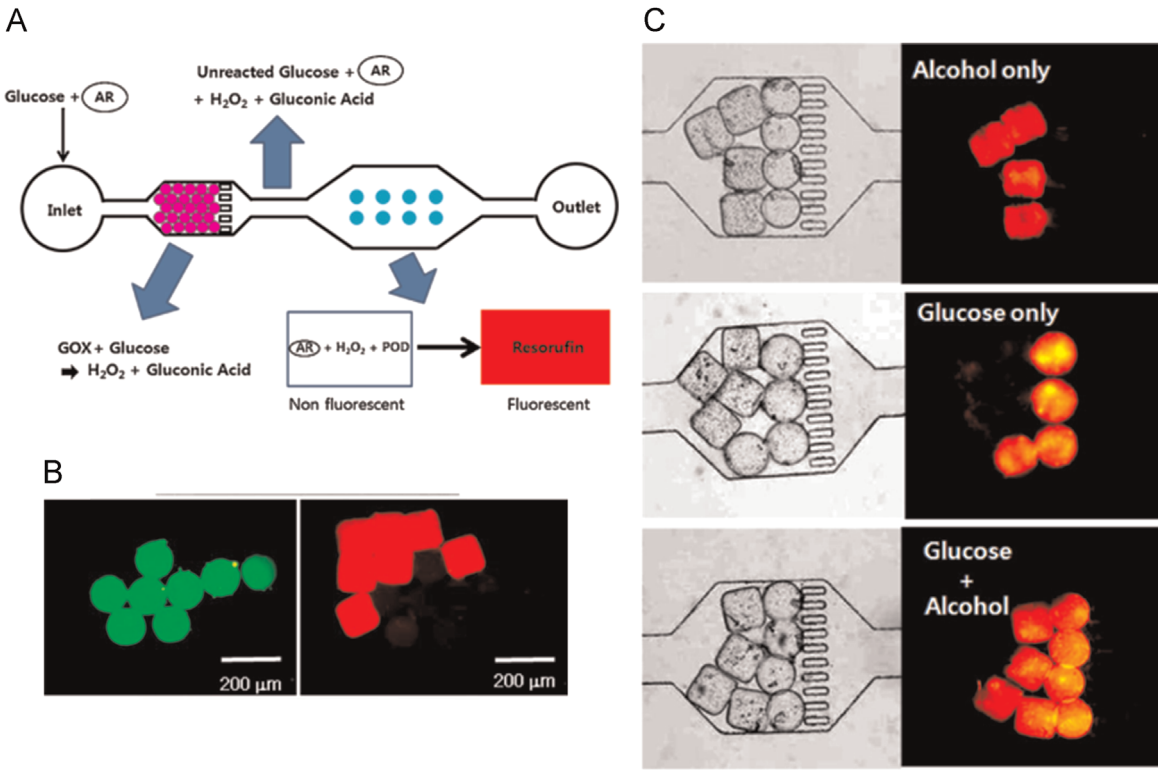

Fig. 8. Two-step reactions and parallel sensing using microparticles: (A) sequential chambers, adapted from Kim et al. (2009), Copyright 2009, with permission from Elsevier, (B) different colors, adapted from Choi et al. (2008), Copyright 2008, with kind permission from Springer Science and Business Media and (C) different shapes, adapted from Jang and Koh (2010), Copyright 2010, with permission from Elsevier.

an albumin matrix to form a glucose responsive membrane sealing an insulin reservoir (Chu et al., 2012). When the membrane came into contact with glucose solution, the gluconic acid produced in the glucose oxidase reaction lowered the pH in the membrane, causing the PNIPAM-co-PMAA nanoparticles to shrink and thereby open pores in the membrane. The device was implanted into diabetic rats and was able to release insulin from its reservoir depending on the glucose level of the animals.

Typical detection techniques employed in systems with microparticles are either amperometry (Godino et al., 2010; Hernandez et al., 2013; Ito et al., 2007, 2011; Sheng et al., 2012) or optical techniques such as fluorescence measurements (Jang and Koh, 2010; Kim et al., 2009). The detection limits and linear ranges for these methods are in the same range as those reported in Section 3, making the immobilization strategies comparable in terms of measurement performance.

5. Enzymes on paper and on thread

5.1. Fabrication

With features like low cost, suitability for mass fabrication methods (e.g. roll-to-roll processing, printing), light weight and easy disposal, paper is considered to have great potential for the large-scale fabrication of cheap and widespread point-of-care devices (Yetisen et al., 2013). A great variety of microfluidic systems has been constructed from paper, ranging from simple devices with one channel to sophisticated origami designs (Liu and Crooks, 2011). Among different biorecognition elements, also enzymes have been integrated with paper-based microfluidics. In its simplest form, paper acts solely as an immobilization support which is integrated into a system made from other materials (Tan et al., 2012). Here, the focus will be on systems which are entirely made from paper.

The paper most often used for microfluidic enzymatic systems is cellulose-based filter paper, e.g. Whatman filter paper (Maattanen et al., 2011). Due to its high permeability and the associated capillary effect, applied liquids penetrate it and spread without the need for actuation (Maattanen et al., 2011). Microfluidic features can be defined on paper by rendering certain areas of the hydrophilic material hydrophobic so that applied liquids only spread into the hydrophilic areas. Materials for the patterning of paper include wax (Lu et al., 2009; Nie et al., 2010), PDMS (Maattanen et al., 2011), alkyl ketene dimer (Li et al., 2010b), polystyrene (Abe et al., 2008) or SU-8 (Chen et al., 2012; Dungchai et al., 2010). Except for SU-8, all of these materials can be applied and patterned by inkjet, flexographic or wax printing, thereby facilitating mass fabrication methods. If such reagents have to be avoided, the material can also be cut into desired structures using, for example, a knife plotter (Yu et al., 2011). In order to seal the cut structures and produce an entire system, these have to be placed between two layers of material, e.g. adhesive tape.

Recently, thread has emerged as a new material for microfluidic devices which shares many advantageous properties with paper, such as low cost, light weight, suitability for mass fabrication methods like sewing or knitting and liquid actuation through capillary action (Li et al., 2010c; Reches et al., 2010). In comparison to paper, it has greater tensile strength, can easily form three dimensional structures and can easily be incorporated into garment (Nilghaz et al., 2013). Typical thread materials are cotton (Li et al., 2010c; Xun et al., 2015; Reches et al., 2010) and polyester (Yu-An et al., 2014), the former being hydrophobic in its natural state and thus has to be plasma treated (Li et al., 2010c) or mercerized (Reches et al., 2010) in order to wick fluids. Microfluidic pathways are either built by single threads (Fig. 9D) which can be stitched through supporting films or attached to adhesive tape or by using woven fabrics with hydrophilic paths surrounded by hydrophobic

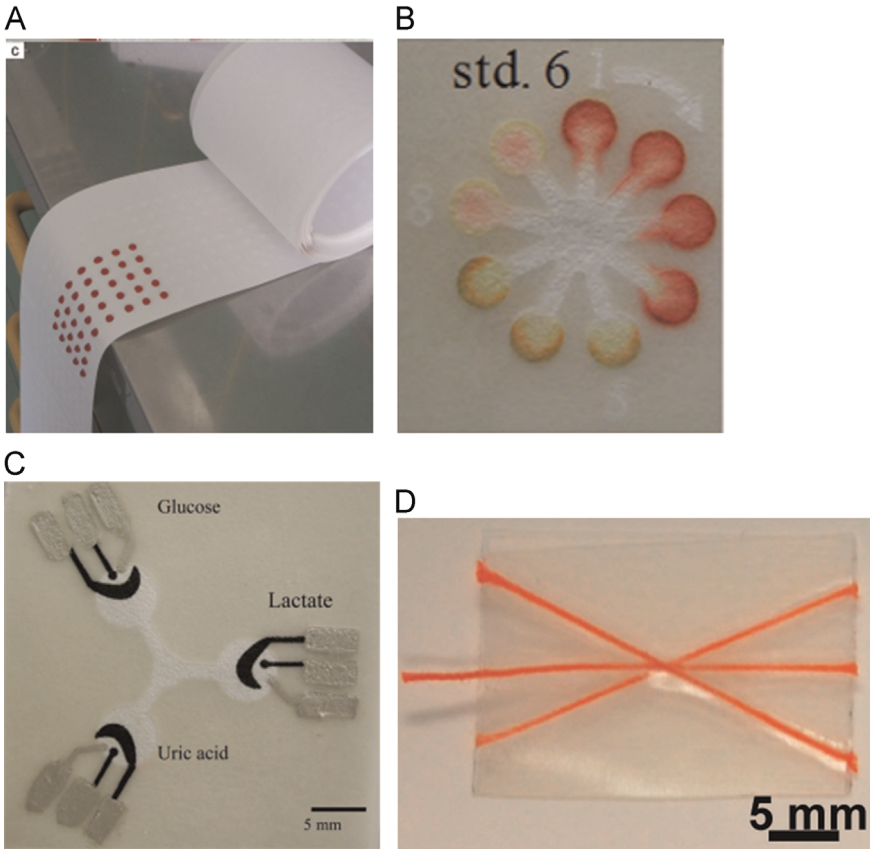

Fig. 9. Paper- and thread-based devices: (A) microtiter array, adapted from Maattanen et al. (2011), Copyright 2011, with permission from Elsevier, (B) different color intensity for the same analyte, adapted from Dungchai et al. (2010), Copyright 2010, with permission from Elsevier, (C) electrodes on paper (Dungchai et al., 2009), Copyright 2009, reprinted with permission from American Chemical Society and (D) woven threads, adapted from Reches et al. (2010), Copyright 2010, with permission from American Chemical Society.

areas (Nilghaz et al., 2013). For further insight into thread-based microfluidics, the reader is referred to reviews dedicated to the subject (Ballerini et al., 2012; Tomazelli Coltro et al., 2014; Nilghaz et al., 2013).

5.2. Functionalization

Thanks to the high porosity of filter paper (Yetisen et al., 2013), enzymes in aqueous solution can be immobilized by absorption. The disaccharide trehalose can be added to the solution to enhance the dry storage stability of the enzymes (Chen et al., 2012; de Souza et al., 2012; Dungchai et al., 2010). Despite such stabilizing reagents, the storage stability of most systems is limited to a few days.

In parallel with the definition of microfluidic structures, printing methods can also be employed for the application of enzymes. Abe et al. (2008) prepared an enzyme ink by mixing glucose oxidase and horseradish peroxidase with citrate buffer, o-toluidine and ethanol. The solution could be printed on filter paper with an inkjet printer. When glucose oxidase is mixed with starch and polyvinyl alcohol, the solution is well suited for screen printing (Maattanen et al., 2011).

The straightforward immobilization procedure for paper and precise printing methods allow designs with multiple parallel sensing sites. Symmetrical designs with a central inlet and multiple channels branching off are often found, as shown in Fig. 9 (Dungchai et al., 2010; Li et al., 2010b; Lu et al., 2009). Maattanen et al. designed a microtiter array on paper, showing the potential to perform multiple isolated measurements on the same device.

Similar to microfluidics made from paper, thread-based systems can be functionalized with enzymes at specific locations by simple absorption of dispensed enzyme solutions (Yu-An et al., 2014). An interesting alternative is to string polyacrylamide particles incubated in enzyme solution onto a cotton thread (Reches et al., 2010). The polyacrylamide particles preserved the activity of the enzymes glucose oxidase and horseradish peroxidase for five days.

5.3. Characteristics and performance

As most paper-based systems are meant to be used in environments without sophisticated laboratory equipment, detection methods operating with simple or no electronic equipment are preferable. Many systems therefore rely on colorimetry which transfers the enzymatic reaction into a color change of the sensing site. Readout of the color change and comparison with a calibration chart is possible with a CCD camera or an office scanner (Abe et al., 2008; de Souza et al., 2012; Maattanen et al., 2011) or by the naked eye (see Subsection 2.4.3.) (Dungchai et al., 2010; Lu et al., 2009). While readout by eye does not require any measurement equipment and enables measurement ranges up to around 30 mM for glucose (Abe et al., 2008), it also has a very limited accuracy which only allows determining approximate concentration ranges for analytes. To enhance the accuracy, multiple sensing sites for the same analyte can be used (Dungchai et al., 2010). Different mixtures of color indicators make the sites show different colors and intensities for the same concentration, allowing untrained users to determine the concentration range more precisely in comparison to a single color reaction. A related approach is the integration of multiple sites for calibration which however

necessitates the availability of calibration solutions (Li et al., 2010b).

It should be noted that detection of substances in whole blood using optical measurement techniques is difficult to achieve due to the strong color and opacity of the blood (Chang et al., 2011; Maattanen et al., 2011). Measurements therefore have to be done in blood serum or other body fluids such as urine or saliva have to be used.

Besides optical detection principles, also amperometric measurements have been realized on paper (Fig. 9C) (Dungchai et al., 2009; Nie et al., 2010). In comparison to colorimetric detection, where color development can take up to 30 min (Chen et al., 2012), amperometry delivers faster and more precise results but requires integrated electrodes and electronic equipment for readout. Consistent with the fabrication principles described so far, the electrodes can be produced by screen printing. Working and counter electrodes can be made from graphite paste, possibly with incorporated redox mediators such as Prussian Blue (Dungchai et al., 2009). As silver chloride ink suited for screen printing is commercially available, also a stable reference electrode can be produced in this manner. Nie et al. designed their device in such a way that it could be connected to and read out by a commercial glucometer (Nie et al., 2010). Microfluidic systems for the amperometric detection of glucose, lactate, cholesterol or alcohol were produced, demonstrating the extension of an established technology platform to new analytes and another possibility for a simple and cheap detection method for paper-based devices.

Paper-based devices with integrated electrodes are also well suited to measure cancer markers. Zang et al. designed a two layered device with working electrodes produced by screen printing on the first layer and a common reference and counter electrode on the second layer (Zang et al., 2012). The working electrodes were modified with multi-walled carbon nanotubes and functionalized with capture antibodies for the cancer markers r-fetoprotein, carcinoma antigen 125, carcinoma antigen 199 and carcinoembryonic antigen. For detection, a sandwich incubation with the cancer markers and antibodies labeled with horseradish peroxidase was performed. The addition of o-phenylenediamine and H_2O_2 initiated an enzymatic reaction which was detected by differential pulse voltammetry and gave an increasing response with increasing cancer marker concentration. In a further development of the approach, the working electrodes were modified with graphene to accelerate electron transfer and the sandwich incubation enhanced by using functionalized silica nanoparticles to detect cancer markers in the range of pg/ml (Wu et al., 2013). Recently, a new method for signal amplification in cancer marker detection has been demonstrated which utilizes radical polymerization to introduce numerous binding sites for horseradish peroxidase at the antibodies used in the detection scheme (Wu et al., 2014).

The measurement range for paper-based devices depends on the readout method of the sensor signal. If the signal is detected by a photodetector, detection limits of 50 μM and less for glucose have been shown feasible (Chen et al., 2012; Cocovi-Solberg et al., 2012). When the signal is read out by eye, a detection limit of 0.5 mM for glucose is feasible (Dungchai et al., 2010). Linear ranges for colorimetric detection go up to around 30 mM for glucose (Abe et al., 2008) while other techniques such as chemiluminescence allow linear measurements up to 50 mM (Yu et al., 2011).

6. Systems with enzyme solutions

6.1. Mixing

The immobilization of enzymes in a microfluidic system adds complexity to the fabrication process and makes regular renewal of the enzyme layer or the entire system necessary. To circumvent these issues, the enzyme can be directly introduced into the system as a solution at the time the measurement is taking place. Systems with enzyme solutions require efficient mixing of enzyme and sample solution before the measurement can take place. Since flow in microfluidic channels is laminar, the simplest form of mixing is to merge streams of the different solutions into the same channel where they mix *via* lateral diffusion (Fig. 10A). The microfluidic design therefore has to include structures like meander-shaped channels where the mixing takes place (Grabowska et al., 2007; Ordeig et al., 2012). Computational fluid dynamics simulations can be carried out to determine the minimum channel length required for complete mixing at a given flow rate (Wu et al., 2007). To accelerate the process, features like herringbone grooves (Moon et al., 2010) or arrow-head shaped baffles (Fig. 10C) (Songjaroen et al., 2009) which create turbulences can be integrated into the channels. In systems dedicated to electrophoretic measurements, also the different electrophoretic mobility of species and sequential injection schemes can be employed for mixing (Atalay et al., 2012; Chun-Mao et al., 2007). Substances with higher mobility in the electrophoresis channel overtake those with lower mobility and mixing takes place (Fig. 10B).

After mixing, the enzymatic reaction occurs before the generated product is transported to the detector located downstream. The length of the mixing channel and the flow rate in it determine the reaction time which has strong influence on signal strength, linear range and sensitivity of the measurement (Demirkol et al., 2011; Ohnishi et al., 2010; Songjaroen et al., 2009; Wu et al., 2007). For example, for absorbance measurements an optimum reaction time for the enzymatic reaction can be found that gives the strongest color development and hence the highest sensitivity (Wu et al., 2007). In the colorimetric detection of creatinine in urine *via* the (nonenzymatic) Jaffé reaction, a too long reaction time can lead to interferences caused by other substances present in the sample (Songjaroen et al., 2009).

For further information regarding mixing in microfluidic systems, the reader is referred to reviews dedicated to the subject (Capretto et al., 2011; Lee et al., 2011; Suh and Kang, 2010).

6.2. Actuation

Another characteristic of systems with enzyme solutions is the wide variety of methods employed for actuation and control of the fluids. While these aspects have to be taken into account for every microfluidic system, they are especially important for systems described in this section since at least two solutions have to be moved through the system, mixed, measured and guided to an outlet.

Lab-on-CD is a specific class of microfluidic systems which makes use of the centrifugal force to actuate fluids (Fig. 10F). This method was also combined with enzymatic sensing (Vengasandra et al., 2010). Microfluidic structures including channels, reagent and reaction chambers and burst valves were embossed into a foamed polypropylene disc and sealed with a silicone rubber layer. The disc together with electronics for photoluminescence sensing could be spun in a standard CD drive which caused the burst valves to open and release the enzyme solutions contained in the reagent chambers into the reaction chambers with the analyte solution. The disc was divided into four parts so that glucose, lactate, ethanol and dissolved oxygen could be detected simultaneously.

In droplet-based or digital microfluidics, droplets instead of constant fluid streams are moved along channels or paths (Fig. 10E). Actuation of the droplets is often based on electrowetting (Hadwen et al., 2012; Srinivasan et al., 2004) but liquids can

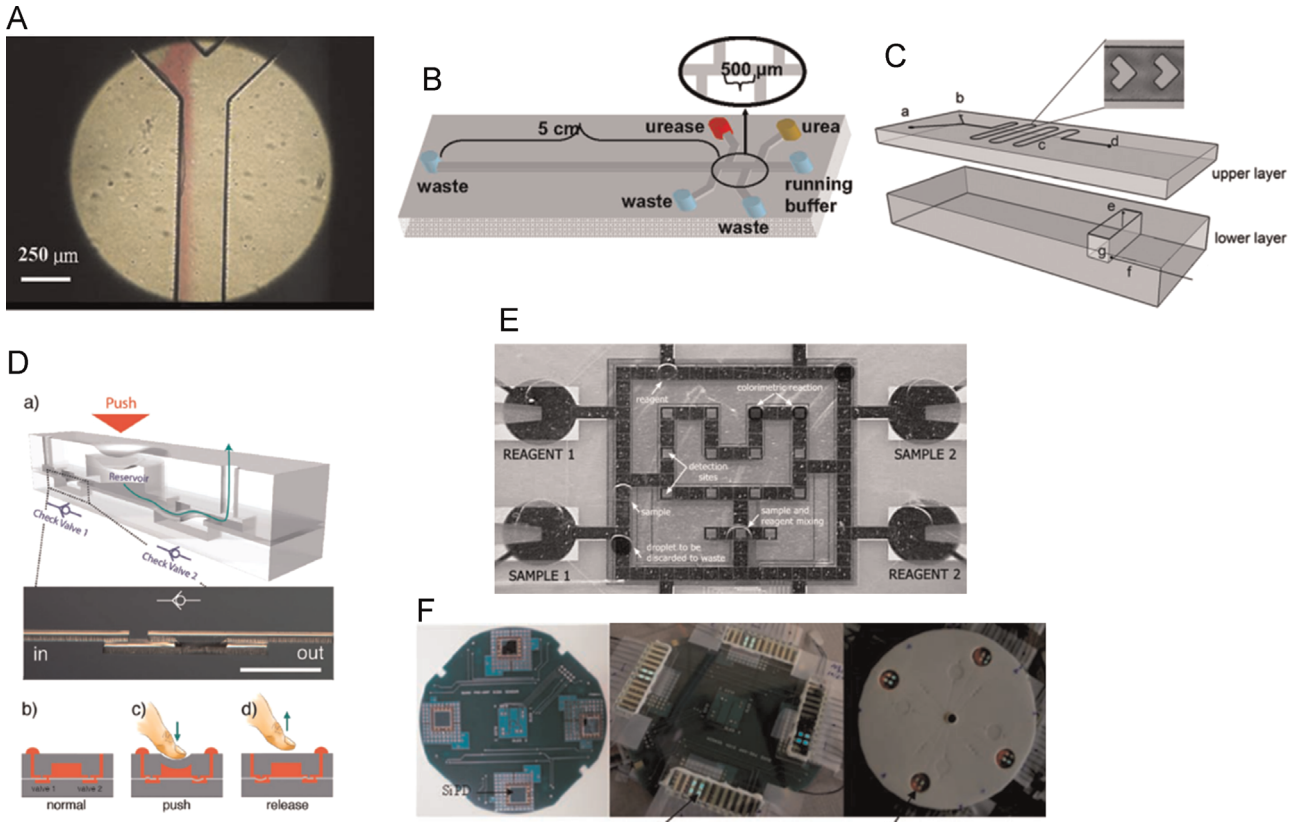

Fig. 10. Mixing in microfluidics: (A) Y-shaped merging, adapted from Wu et al. (2007), Copyright 2007, with kind permission from Springer Science and Business Media, (B) different electrophoretic mobility, adapted from Chun-Mao et al. (2007), Copyright 2007, IEEE, reprinted with permission, (C) arrow-head shaped baffles (Songjaroen et al., 2009), Copyright 2009, reprinted with permission from Elsevier; Actuation of solutions, (D) by thumb pressure, adapted from Li et al. (2012) with permission of The Royal Society of Chemistry, (E) in digital microfluidics (Srinivasan et al., 2004), reprinted with permission of The Royal Society of Chemistry and (F) in lab-on-CD (Vengasandra et al., 2010), reprinted with permission of The Royal Society of Chemistry.

also be mixed with iron particles and moved along the surface by magnetic fields (Lindsay et al., 2007). The small volume of the droplets down to the nanoliter range allows rapid mixing and low reagent consumption. When combined with large electrode arrays, a very high degree of flexibility in the manipulation of the droplets can be achieved, allowing splitting, merging and mixing operations (Hadwen et al., 2012). On the downside, the systems need complex control logic and a filler medium such as dodecane (Hadwen et al., 2012) or silicone oil (Srinivasan et al., 2004) to prevent evaporation of the small droplets.

A system operated by thumb pressure for the colorimetric measurement of glucose or uric acid was developed by Li et al. (2012) (Fig. 10D). The system made from multiple layers of soft PDMS contained reservoirs, check valves, metering channels and a reaction chamber. By squeezing the reservoirs sequentially, analyte solution and colorimetric reagent solution could be pumped through the system and defined amounts of the solutions were metered. Finally, the solutions were brought together in a reaction chamber where mixing and enzymatic reaction took place. The resulting color change was interpreted by eye, allowing measurements up to 10 mM for glucose concentration. Another way to avoid external pumps is the usage of hydrogen bubbles produced at electrodes in the microfluidic system (Satoh et al., 2007). H_2 bubbles formed by electrolysis of an electrolyte in special compartments can deform soft PDMS layers and displace and drive fluids contained in neighboring channels.

6.3. Characteristics and performance

Using enzyme solutions avoids the problems of reduced activity and steric hindrance which may occur when enzymes are immobilized (C. Wang et al., 2010a). Fresh enzyme with the same activity is introduced for every measurement, possibly giving more reproducible measurements. Enzyme solutions can also be enriched inside the microfluidic system, allowing measurements with different enzyme concentrations (C. Wang et al., 2010a). Furthermore, ready-made enzyme assay kits for optical detection are typically available in solution form, including more unusual analytes like non-esterified fatty acids (Clark et al., 2010) or glycerol (Clark et al., 2009).

On the other hand, there are some drawbacks regarding this type of enzyme handling. The enzyme solution has to be supplied to the system in some way, making internal or external reservoirs necessary. The amount of enzyme needed for measurements is bigger than that of immobilized enzymes, at least when several measurements are done with the same system. At the same time the amount of waste produced by the system increases.

7. Summary and conclusions

The combination of microfluidics and enzymatic sensors with today's variety of designs, sensing principles and immobilization methods has opened up the possibility to measure a broad range of analytes but also requires a profound knowledge in several scientific areas. The question every proposed system has to face up to is whether it has the potential to make the transition from a proof of concept to a successful and widely employed product – be it for application in point-of-care testing, in laboratories for scientific research or in resource-poor environments. The systems

discussed in this review with their great variety of features show potential for all of these fields. However, the choice of an appropriate combination of microfluidic design, enzyme integration and detection principle is crucial to achieve the required performance for the targeted application.

For applications in point-of-care testing, precise and fast measurements are required (Price, 2001; Yager et al., 2008). The system should be portable; however a certain range of additional equipment such as small readout electronics is acceptable in environments such as doctor's offices or in home care. It is envisioned that systems for the parallel measurement of multiple substances will have the greatest impact in these environments since for the reliable diagnosis of diseases often multiple indicators have to be measured (Spindel and Sapsford, 2014). Electrodes seem to be the ideal enzyme support in this context, as these offer fast and precise measurements in combination with amperometry and fit well into microfluidic designs for parallel measurements. If reusability of the system is a requirement, electrodes can be combined with microparticles, because these extend the lifetime of a device by offering a way for easy renewal of the functionalization. This approach also helps lowering the application costs and thereby may help raise acceptance for a new technology.

For applications in research or hospital laboratories, measurements with high sensitivity and low detection limits are of importance. Here, the combination of enzymes on microfluidic channels and optical readout is an interesting option since optical methods offer low detection limits down to the nanomolar range. The expensive and bulky equipment needed for high precision optical detection makes this approach more suitable for use with benchtop instruments than for point-of-care applications. If a new measurement has to be set up fast and enzyme immobilization has to be avoided, enzyme solutions can be used. Efficient mixing of solutions and precise control of reaction time are two critical points in this approach. Lab-on-CD and systems operated by thumb pressure might be interesting concepts with enzyme solutions which could make the transition into the point-of-care market.

Paper has the biggest potential for cheap, single use enzymatic systems due to its low cost for material and fabrication. In combination with colorimetric detection, the measurement signal can be read out by the unaided eye, making additional equipment for readout unnecessary. Although the accuracy of these systems falls behind systems with sophisticated electronics, paper-based microfluidics could be among the first to reach widespread distribution as point-of-care devices. Issues such as long measurement times and the lacking suitability for whole blood have to be tackled, however.

8. Future perspectives

While possible applications for enzymatic microfluidic systems are widespread, some major problems have to be solved in order to reach commercial maturity. One is the widely varying stability and lifetime of the systems. The reported storage stability ranges from a few days to more than 12 weeks, depending on immobilization method, storage conditions and number of measurements conducted. A shelf life of at least one month is highly desirable, especially for applications in third world countries. A related issue is the stability of the measurement signal in devices meant for multiple consecutive measurements. The signal decay in these devices over time should be as low possible to minimize the number of calibrations necessary.

In the fabrication of microfluidics with integrated enzymes, an optimum method for the leak-proof assembly of functionalized parts is yet to be found. Too many systems rely on reversible clamping, which might be a practical solution for research but does not provide a reliable connection, if systems are to be packed, shipped and used by untrained users. Functionalization after assembly of the system provides a solution to this problem but at the same time complicates the immobilization procedure. Also, for the fabrication in large quantities, batch methods for functionalization are necessary. Microparticles and printing methods seem to be the best choices in this respect.

After these problems have been solved, microfluidic enzymatic sensor systems have the potential to become a new class of fast, affordable and versatile measurement equipment that could change the way measurements are done in the health care sector.

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