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Chemical Surface Modifications for the Development of Silicon-Based Label-Free Integrated Optical (IO) Biosensors. A review.

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ABSTRACT. Increasing interest has been paid to label-free biosensors in recent years. Among them, refractive index (RI) optical biosensors enable high density and the chip-scale integration of optical components. This makes them more appealing to help develop lab-on-a-chip devices. Today, many RI integrated optical (IO) devices are made using silicon-based materials. A key issue in their development is the biofunctionalization of sensing surfaces because they provide a specific, sensitive response to the analyte of interest. This review critically discusses the biofunctionalization procedures, assay formats and characterization techniques employed in setting up IO biosensors. In addition, it provides the most relevant results obtained from using these devices for real sample biosensing. Finally, an overview of the most promising future developments in the fields of chemical surface modification and capture agent attachment for IO biosensors follows.

Contents

1. Introduction. Approach to Refractive Index Optical BioSensors

Nowadays, biosensing is a scientific and technological hot topic given its potential in fields such as medical diagnosis, healthcare, environment, defense and food security. In these realms, the specific and sensitive detection of targets in short-time analyses plays a primordial role.

Traditionally, labeled formats have been used, where targets or reporter molecules carry fluorescent, enzymatic or radioactive tags. These techniques present high sensitivity, and even achieve single molecule detection [1], and are currently the standard techniques for many determinations. However, the development of label-free techniques has attracted the interest of many researchers over the last decade [2-6]. They offer advantages such as direct detection, real-time monitoring, kinetic information, fewer reagent costs, and the native molecular conformation of the protein is not altered by a tag. Thus, label-free biosensors based on optical [7], electrical [8-13] and mechanical principles [14-18] can be found. Optical sensors are more versatile than others because they can be made from different materials, such as silicon, glass, metals or polymers, and they offer different detection modes and architectures that can be combined [19]. They also offer other advantages; mass-scale fabrication, excellent physical properties, good selectivity and sensitivity; and can accomplish multiplexed detection in a single device [20,21]. Label-free optical biosensors have received increasing attention and many reviews can be found that provide a general overview of the state of the art [22-25]. In label-free optical detection, the transduction mode may be based on the refractive index (RI), optical absorption or Raman spectroscopy [26-30]. In past two decades, optical sensors based on refractive index (RI) changes feature among the most studied (Figure 1).

In ordinary dielectric material, the refractive index (RI) directly relates to the polarizability of molecules at optical wavelengths. Biological molecules have a higher RI than air or water, and they lower the propagation speed of the electromagnetic fields passing through them. Optical biosensors are designed to translate changes in the propagation velocity of light through a medium that contains biological material into a quantifiable signal proportional to the amount of material present on the sensor surface. For this reason, the electromagnetic field bound to an optical device that couples some energy to an external medium (called an evanescent field) penetrates a few hundred nanometers into the optically rarer environment from the optically denser guiding medium.

Different optical phenomena have been employed to design RI optical biosensors. Representative methods include: Surface Plasmon Resonance [31,32], Reflectometric Interference Spectroscopy [33,34], Dual polarization Interferometry [35,36], Photonic Crystal Technology [37,38], and Whispering Gallery Mode Resonators [39,40]. Extensive reviews have been written and detail all these approaches which have developed to act as biosensors [25, 39, 40, 41].

The search for analytical platforms that operate rapidly and efficiently has received increasing interest in recent years, not only for RI optical biosensors in particular, but also for label-free biosensors in general [42,43]. Optical label-free biosensors are ideal candidates for lab-on-a-chip (LOC) applications [41,44,45] as they allow the integration of both fluidic handling and optical analyses into a single chip. These integrated sensing devices enable the mass production of high-density biosensors, and provide rapid, sensitive and multiplexed measurements at the required point. Integrated Optical (IO) Biosensors employ guided waves or modes in planar optical waveguides. Besides Silicon-on-Insulator (SOI) technology, waveguide materials usually include high

90   refractivity silicon dioxide or titanium dioxide and silicon nitride films in oxidized

91   silicon wafer substrates.

92   A number of different IO biosensors has been designed and significant advances have

93   been made in label-free interferometric [46], grating-coupled [47,48], photonic crystals

94   [37,38,39] and microcavity resonators [39,40,49-52] biosensors.

95   Within waveguiding interferometers, Mach-Zehnder [44,53-55], and in a less extent

96   Young interferometers [56,57] are used mainly for biosensing, although novel

97   interferometric designs with improved performance are being continuously explored

98   [58-60].

99   The materials used to construct integrated interferometers for biosensing include

100   mainly silicon oxide, silicon nitride, SOI and, to a lesser extent, polymers.

101   Grating coupled sensors are made from SiO_2, Ta_2O_5 and SiO_2/TiO_2 on glass

102   substrates, and they rely on the phenomenon that the coupling of light of a certain

103   wavelength into a planar optical waveguide via grating occurs only at a critical

104   incidence angle. The measurement principle can be Optical Waveguide Lightmode

105   Spectroscopy (OWLS) [47] or Wavelength Interrogated Optical Systems (WIOS)

106   [61,62].

107   Photonic crystals (PhC) are dielectric structures whose periodicity is in the order of a

108   wavelength. The frequency of light that is coupled into PhC depends on the RI in the

109   local environment around an introduced defect, which acts as a transduction signal

110   when biorecognition takes place [63]. In other designs [38,64], binding events shift the

111   wavelength of the reflected light proportionally to the adsorbed mass.

112   Another emerging class of miniaturized optical resonators, which reach exceptionally

113   high-quality Q-factors, are the Whispering Gallery Mode resonators [65]. To date, they

114   have been implemented into three major configurations: microfabricated rings, disks

115 and toroids [39,41], stand-alone microspheres [66,67], and capillary-based optofluidic

116 ring resonators (OFRs) [68]. Among them, silica micro disks, rings and toroids are

117 preferred for IO devices.

118 An excellent overview on the recent developments in resonant microcavities and

119 photonic crystals for chemical and biological analysis was recently published by

120 Luchansky and Bailey [39].

121 Other highly sensitive label-free biosensors based on optical fibers also exist [69].

122 However, they need external optical components that are not integrated into the chip, as

123 well as larger sample volumes. Therefore, miniaturized optical devices are preferred for

124 portable applications that also include LOC architectures.

125 Two points are key to construct IO biosensors: optofluidic integration [25,39,41,70-

126 72] and device biofunctionalization. Regarding functionalization, and although

127 everyone agrees on the importance of proper surface functionalization to provide

128 selectivity and good sensitivity, systematic studies into the biofunctionalization

129 processes employed in these devices are lacking. Such methodologies are generally

130 based on the same principle, but there is disagreement about procedures, and treated

131 surfaces are often poorly characterized. Because silicon technology appears to be the

132 choice currently preferred for the majority of IO biosensors, the biofunctionalization

133 chemistries employed in the sensors constructed on silicon-based materials are

134 discussed in the next section.

135 Surface modification of planar silicon-based substrates, with covalently linked

136 organic monolayers, has been extensively studied [73-76]. In addition, the literature

137 describes organic surface modification of silicon nanowires for sensing [77,78].

138 This review is an overview of all the different surface modification strategies

139 explored to functionalize Si-based IO biosensors, and it discusses the bioreceptors and

140   methodologies employed, the techniques available for surface characterization, and the

141   main achievements accomplished in real biosensing.

142

2. Surface Chemistry Approaches for Bioreceptor Attachment on Silicon-Based Materials

2.1. Chemical Surface Modifications and Bioreceptors Attachment

The specific detection of analytes is based on the immobilization of a bioreceptor that interacts with the target of interest. Immobilization can be done in two ways: direct adsorption; covalent, electrostatic or affinity binding. In all cases, it is necessary to modify the surface of the support material to the extent that the material properties are tuned to accomplish the best analytical characteristics. Performance comprises: favoring the receptor attachment that induces selectivity to a target of interest; preventing surface fouling; changing the hydrophobicity/hydrophilicity of the surface, while maintaining the sensing system's physical (optical, mechanical, etc.) properties.

Although bioreceptor physisorption has been widely used, especially for the preliminary demonstrations of new optical sensors designs [79-87], it has several drawbacks, which include: random orientation, lack of reproducibility, long incubation times, and risk of folding and desorption when the receptor is adsorbed. This issue is very important when working in flow and when chip regeneration is desired. In such cases, the covalent attachment of receptors is recommended.

Ideally, biofunctionalization chemistries should fit the following requirements: gentle enough to avoid the structural damage of both the receptor and the transducer; few reaction steps; low optical adsorption at the working wavelengths; homogeneously thin layer formation that is compatible with evanescent field sensing; good surface coverage; reproducibility; robustness; low non specific binding; minimal sample and reagents consumption; easy handling; biocompatible conditions (pH, ionic strength, solvent, etc.); integrability with mass-scale fabrication.

167   Selecting a proper immobilization technique is a key point as many factors can

168   negatively affect final biosensor performance. Several aspects, such as orientation and

169   probe density on the surface, pH, target concentration, matrix effects, operating

170   conditions and impact of the targeting strategy on transducer sensitivity, must be

171   carefully analyzed.

172   Figure 2 represents the main functionalization approaches employed to construct IO

173   biosensors.

174   *2.1.1. Chemical Surface Modification by Self-Assembled Silane-Based Layers*

175   Most of the methods applied to functionalize silicon surfaces employ the self-

176   assembly of organofunctional alkoxysilanes (Figure 2a). This strategy assumes standard

177   glass-based surface functionalization chemistry, and is well-suited to the

178   functionalization of silica-on-silicon optical devices. The reaction is based on the

179   condensation between the siloxanes of the organosilane and hydroxyl moieties present

180   on the surface. Thus, the density of silanol groups is a determinant to form a proper

181   organic layer. In the case of silicon and silicon nitride materials, the hydroxyl groups

182   derive from the native silicon oxide layer, which is always present, although etching the

183   native layer and forming a new one, usually by thermal oxidation, is commonplace [88-

184   92].

185   The formation of silane self-assembled monolayers (SAMs) is more complex than the

186   assembly of thiol molecules on gold surfaces. Yet it offers one important advantage in

187   that silane-terminated monolayers show higher physical and chemical stability as

188   opposed to thiol-ended ones. Therefore, it is possible to apply a large pool of chemical

189   reactions. Alkylsiloxane monolayers are usually prepared by a chemisorption process of

190   self-assembling molecules, such as trichloro-, trimethoxy- or triethoxysilanes, onto the

191   solid substrate [93]. Despite the formation mechanism having been extensively

192   investigated, there is still some controversy [94-95]. It is well-known that certain

193   parameters, such as water content, solvent use, age of the solution, deposition time and

194   temperature, are still largely depended on [96]. In our opinion, not enough attention is

195   paid to these issues. Frequently, the reaction conditions are established randomly, as

196   evidenced by the fact that different concentrations, solvents (from aqueous ethanol to

197   anhydrous toluene), reaction times or temperatures are employed for the same

198   organosilane, by assuming that a monolayer is formed.

199   Immediately before silanization, surfaces are cleaned with oxidant media to remove

200   organic pollutants and to increase the hydroxyl moieties on the surface ($\approx10^{15}$ per cm^2)

201   [73]. A cleaning process to generate reactive hydroxyl groups is critical for the effective

202   immobilization of silanes. There are several types of Si-OH groups that can be formed

203   on silica surfaces. Some (germinal and isolated silanols) are reactive, whereas others

204   (the vicinal silanol and siloxane groups) are not. The most widely used oxidants are

205   oxygen plasma [97-103] and piranha solution [63, 104-111], consisting of a

206   concentrated sulfuric acid: a hydrogen peroxide mixture at different ratios varying from

207   3:1 to 7:3. This treatment is well-performed at room temperature or by heating, but

208   usually for only a few minutes. The literature also describes other oxidants and cleaning

209   agents comprising ozone-UV [112], sodium hydroxide [113], the ammonia:hydrogen

210   peroxide mixture [114,115], nitric acid [53,116], hydrochloric acid [117], sulfuric acid

211   [118], chromic acid [119], or mineral acids with hydrogen peroxide [115]. Sometimes

212   more than one of these treatments is combined and sequentially applied to the chip

213   [115,117,120,121].

214   Our group compared the two mostly used oxidation protocols -oxygen plasma and

215   piranha treatment- using planar silica chips. In both cases, the water contact angle

216   became 0º after the oxidation step (36º before oxidation), indicating the large number of

217   hydroxyl groups created on the surface. Afterward, the organosilane layer formed using

218   3-aminopropyltriethoxysilane (APTES) provided similar results for both procedures.

219   Piranha solution was also used to regenerate the surface. However, our experience

220   indicates that the best procedure must be evaluated in each case; thus, we found the

221   piranha treatment was the most suitable for silicon and silicon nitride materials, while a

222   chromic mixture was the best choice for silicon oxynitride surfaces. Furthermore, the

223   number of feasible regenerations on the same surface is limited and has to be evaluated

224   experimentally. Our studies have found that the number of regeneration cycles ranges

225   from three to five.

226   Among the vast variety of commercially available organosilanes [122], very few have

227   been used to functionalize IO transducers. In our opinion, there are two reasons for this:

228   first, short alkyl chains are preferred as the evanescent field decays with distance from

229   the surface; second, the methods are adopted from the well-established glass-based

230   bioconjugation methods employed in biochips. Thus, $-NH_2$, -SH, -COOH, or epoxy

231   functionalities, are mainly employed [123].

232   The methods applied for the biofunctionalization of silicon-based IO biosensors are

233   discussed according to the terminal functionality of the organosilane used. The

234   bioconjugation protocol following organosilane layer assembly is also critically

235   presented.

236   *NH_2 organosilane*

237   Given its reactivity to aldehyde, carboxylic acid and epoxy functionalities, APTES (3-

238   aminopropyltriethoxysilane)   [53,63,89-92,   107-110,97,   100,103,   112,

239   114,116,120,124,125-127]   and   APTMS   (3-aminopropyltrimethoxysilane)

240   [88,111,98,99,128] have become the most widely used linker compounds for

241   biofunctionalization purposes (Figure 3). However, the conditions employed differ for

242 silane concentration, solvent and incubation time. Moreover, a curing process is often

243 performed after silanization. The trimethoxy compound is more reactive and can be

244 deposited on a substrate using 100% pure organic solvent. The advantage of this process

245 is that a thinner, and a more controlled deposition of the silane, can be generated to

246 create a monolayer of the aminopropyl groups on the surface. For triethoxysilane, the

247 reaction must occur in the presence of water, otherwise the ethoxy groups are not

248 reactive enough to spontaneously couple to the hydroxyl groups on the surface.

249 Given the possibility of hydrogen bond formation between the amine of APTES and

250 the SiO_x surface, both the head and tail groups in the organosilane can be oriented

251 toward the surface, which can result in a disordered layer [126]. Additionally, cross-

252 linking among alkoxysilane units may yield oligomerized silane structures, resulting in

253 rough layers that are thicker than a monolayer. The optimal conditions for solvent-based

254 silanization using APTES have been investigated on planar surfaces [96]. Experiments

255 with a 1% APTES concentration provide good films where the reaction time was less

256 than 1 h, and the APTES film becomes thicker with longer reaction times.

257 Having aminated the surface, different procedures are employed to attach the probe.

258 An aminated surface is used to directly immobilize antibodies by adsorption

259 [53,125,127] or to covalently attach an N-hydroxysuccinimide ester-ended biotin (NHS-

260 bt) (Figure 3a) [88,97-100,103,110,111,112,114,116,128]. NHS esters bind in nearly

261 quantitative yields with primary amines, resulting in the formation of a stable amide

262 bond. However, NHS esters typically undergo rapid hydrolysis under aqueous

263 conditions, and functional activity is compromised over time [129]. Hence the quality of

264 the resulting biotinylated surface is highly dependent on the experimental conditions.

265 Guo *et al.* [107] also employed NHS ester chemistry, but differently. An aminated

266 surface is firstly carboxylated by treatment with succinic anhydride (Figure 3b). Then,

267 the active ester is formed with a mixture of 1-ethyl-3-(3-dimethylaminopropyl)

268 carbodiimide (EDC) and NHS. Finally, the succinimide ester surface is used to couple

269 amine-containing proteins, such as streptavidin and bovine serum albumin (BSA).

270   Studies carried out in our lab employing the EDC/NHS conjugation of proteins on

271 APTES-modified silicon oxide planar surfaces have shown that the active ester must be

272 formed under well-controlled conditions; for instance, pH must be 3.5. High sensitivity

273 to the experimental conditions implies that this approach lacks reproducibility and

274 provides low yields of protein immobilization. Frequently, the protein remains on the

275 surface after conventional washings due to passive adsorption. Therefore, assessing the

276 covalent attachment of protein acidic washings is recommended.

277   Aminosilane surfaces are also activated with homobifunctional crosslinkers like 1,4-

278 phenylenediisothiocyanate (1,4-PDI), which provide isocyanate groups that react with

279 amine-ended oligonucleotide probes to form a thiourea bond (Figure 3c) [124].

280 However, the most widely used homobifunctional crosslinker is glutaraldehyde (Figure

281 3d), employed to form an aldehyde-terminated surface which allows the reaction of

282 amine groups by the formation of imines (Schiff bases). By this strategy, antibodies,

283 BSA and amine-ended oligonucleotides have been attached to silicon IO devices

284 [63,89,90,108,114]. Due to the reversibility of the imine bond, some authors have

285 reported the use of a sodium cianoborohydride reduction step to obtain more stable

286 amine bonds [89,108].

287   However, when we attempted this approach on silicon oxide planar surfaces, we

288 observed that when working with a slightly basic pH (9-10), aldolic condensation takes

289 place, providing a short polymer where aminated compounds are covalently attached by

290 single bonds. Thus, reductive conditions are not required to accomplish stable bonds.

291   Another approach which proved robust, reproducible and very suitable for IO

292   biosensors implementation is based on hydrazone bond formation using SoluLink

293   chemistry (Figure 3e) [130,131]. It employs two crosslinkers; 6-hydrazinonicotinamide

294   (S-HyNic) and succinimidyl-4-formylbenzamide (S-4FB); to covalently attach probes

295   on aminated surfaces. This chemistry has been widely used in different configurations

296   for the biofunctionalization of the silicon microring resonators [104,105,132-144]

297   commercialized by Genalyte (to date, the only commercialized device dealing in

298   nanophotonic based biosensing) [145].

299   After accomplishing surface amination with APTMS, 4-polyethylenglycol-4-

300   formylbenzoate (PEG-4FB) is added via succinamide coupling. The probe is previously

301   reacted with a hydrazine nicotinoate (HyNic) moiety by also using succinamide

302   chemistry. Then, a hydrazone bond takes place to covalently link the probe to the

303   surface [132]. The inverse approach can also be used successfully by linking the

304   hydrazine moiety to the surface and modifying the probe with S-4FB [105,133,134].

305   Finally, in order to simplify the number of steps, an organosilane already bearing the

306   HyNic moiety can also be employed to silanize the microring resonator surface

307   [104,135,136,137,138,139,140,141,142,143,144].

308   *SH organosilane*

309   Another approach that also leaves nucleophilic functionality on the surface involves

310   the employment of a thiol-ended organosilane (Figure 4). 3-

311   mercaptopropyltriethoxysilane (MPTS) has been employed by Sepúlveda *et al.* [121]

312   for the functionalization of an integrated Mach-Zehnder interferometer microsystem.

313   This allows a thiolated oligonucleotide to be attached to the surface via disulfide bond

314   linkage (Figure 4a). Thiol functionality also permits the attachment of probes through

315   their amine groups using heterobifunctinal crosslinker m-maleimidobenzoyl-N-

316   hydroxysuccinimide ester (MBS), as demonstrated by Xu *et al.* [119] in an

317   implementation of a planar optical waveguide-based interferometer (Figure 4b).

318   The employment of disulfide bonds to attach thiolated oligonucleotides on silanized

319   surfaces offers the advantage of reusability. As the disulfide bond is reversible, the

320   surface can be regenerated, for instance, by treatment with dithiothreitol (DTT).

321   However, this fact has yet to be demonstrated on an IO device.

322   Another interesting approach is that which utilizes the advantageous click chemistry

323   reaction between thiol and alkene moieties (Figure 4c). Our group has demonstrated the

324   biotinylation of silicon oxide surfaces by this principle to perform hybridization assays

325   in a microarray format, which achieves good performance, and is presently being

326   implemented into a ring resonator-based biosensor [146].

327   *Epoxy organosilane*

328   Epoxy chemistry is an alternative coupling system for biomolecule immobilization

329   given its stability under aqueous conditions and its reactivity to several nucleophiles,

330   such as amine and sulfhydryl groups [147,148]. Thus, surfaces that are covalently

331   coated with 3-glycidoxypropyltrimethoxysilane (GOPTS) can be used to conjugate

332   thiol-, amine- or hydroxyl-containing ligands (Figure 5).

333   GOPTS has been employed to covalently attach antibodies and aminated

334   oligonucleotides by an epoxide ring opening in an optical microring resonator by

335   Ramachandran *et al.* (Figure 5a) [113]. Scheneider *et al.* [118] constructed an IO

336   biosensor based on a Hartman interferometer, performed the oxidation of epoxy

337   moieties to aldehyde groups with sodium periodate, and further proved attachment by

338   reductive amination with sodium cianoborohydride (Figure 5b).

339   In an alternative approach after GOPTS silanization, De Vos *et al.* [101,102] used a

340   thin layer of poly (ethylene) glycol (PEG) to prevent non specific binding in a microring

341 resonator biosensor (avoidance of non specific adsorption is also a critical issue and is

342 dealt with separately). Thus, two heterobifunctional PEGs are used: one bearing thiol

343 and carboxylic acid moieties, and the other containing two amine functionalities, one of

344 them with a protecting group. The use of such reagents allowed the introduction of

345 reactive carboxyl and amino groups onto the surface of the SOI microring. Finally,

346 biotinylation was carried out by EDC/NHS and NH_2-biotin in the first case, and by

347 NHS-biotin in the second [102,110]. Better performance was obtained with the second

348 approach. For the protein attachment on aminated microring resonators,

349 homobifunctional crosslinker di-succinimidyl carbonate (DSC) was also used. However

350 this route proved less efficient than a NHS derivative [101].

351 All the above-mentioned strategies take longer. Recently, we demonstrated the use of

352 epoxy-ended surfaces for the direct attachment of the thiolated oligonucleotides induced

353 by light (Figure 5c). The reaction times are thus cut to a few minutes if compared with

354 the conventional nucleophilic attack of SH [149]. Nucleic acids hybridization assays in

355 the microarray format reveal the potential of this approach which has been employed to

356 develop a microring resonator-based biosensor showing high reproducibility, stability,

357 selectivity and sensitivity to detect hybridized complementary strands with negligible

358 unspecific adsorption (unpublished data).

359 *COOH organosilane*

360 Silane coupling agents containing carboxylate groups have also been utilized to

361 functionalize IO devices with carboxylic acids for the subsequent conjugation with

362 amine-containing molecules (Figure 6a) [58,117]. Duval *et al.* [58] employed

363 carboxyethylsilanetriol sodium salt (CTES) on silicon nitride bimodal waveguide

364 interferometers, and proteins were conjugated to the surface by EDC/NHS. A PhC

365 microcavity sensor was carboxylated by Zlatanovic *et al.* [117] using an effective

16

chelator of metal ions, such as N-(trimethoxysilylpropyl) ethylene-diamine triacetic

acid, while proteins were further conjugated by EDC/NHS. After organosilane layer

formation, the remaining steps were performed online, and no data on yields or surface

characterization are provided, except for the limit of detection for the biorecognition

event.

In addition, carboxylic acid-ended dimethyl monomethoxy organosilane has been

used as a horizontal spacer to form mixed monolayers on a Mach-Zehnder

interferometer by the co-adsorption of binary solutions containing both a carboxyl-

ended organosilane and another bearing biotin moiety [150]. However, there are no

experimental details available on yields, conditions, etc.

EDC/NHS-based chemistry for protein conjugation must be carefully carried out

because reproducibility is highly dependent on the experimental conditions. Protein

attachment to the carboxylated surface can be done in two ways. In a first approach, the

active ester is formed on the surface using an acidic pH (3.5), and the protein is

conjugated to the surface using a neutral pH (6.5). The second way involves the

addition of EDC/NHS together with the protein (pH 6.5), and the conjugation is

performed in one step; this case involves the risk of protein cross-conjugation, giving

rise to aggregates. Besides in both cases, a risk of hydrolysis of the active ester must be

taken into account, and control assays to demonstrate the covalent nature of the link

between the protein and the surface are recommended (the protein can remain on the

surface through the electrostatic interactions between the amine and the carboxylate

moieties, without rendering the advantages of the covalent link).

Isocyanate-ended organosilane

Isocyanatepropyltriethoxysilane (ICPTS) has been used to link proteins onto silicon

photonic crystals without crosslinkers or activation steps being needed [106,151]. The

391 isocyanate moiety reacts with amines to form isourea bonds, and with hydroxyl groups

392 to form urethanes (Figure 6b). Oligonucleotides are also attached to the ICPTS surface

393 using biotinylated probes which are affinity-captured by streptavidin covalently linked

394 to the isocyanate modified surface [151]. This is a simple one-step approach, but the

395 experimental conditions must be well-controlled to achieve an acceptable degree of

396 reproducibility. Thus at a certain basic pH, there is a risk of decarboxylation, which

397 provides an amine-ended surface instead of an isocyanate-ended one.

398 It is noteworthy that organosilane-based chemistries are also being successfully

399 applied to other integrated and non integrated optical label-free biosensors developed

400 with different materials, such as glass microspheres [67] and liquid core optofluidic ring

401 resonators [152,153], planar waveguides made of metal oxides, such as Ta_2O_5 [154,155]

402 or $SixTi_{(1-x)}O_2$ -as in the commercialized OWLS system based on Optical Waveguide

403 Lightmode Spectroscopy [156]-, and on the TiO_2/SiO_2 surfaces of polymeric PhC

404 [19,157,158] commercialized as BIND™ Biosensor by SRU Biosystems [64].

405 Organosilane condensation on the silicon surface can be done by chemical vapor

406 deposition (CVD) [53,97,100,106,111] or by wet chemistry [111,134]. A good

407 comparison between CVD and wet chemistry was made by Hunt *et al.* [111] for the

408 functionalization of silica microtoroids resonators with APTES, who indicated that

409 chemical vapor deposition provides more ordered monolayers.

2.1.2. Other Silicon Surface Chemical Modifications

411 Although silane-based chemistry is the gold standard for the functionalization of

412 silicon-based materials for sensor applications, the literature also describes other

413 alternative surface derivatization approaches for IO biosensors development.

414 Shang *et al.* [159] developed a conjugation strategy based on SAM formation with

415 hydroxyl-ended organophosphonate (Figure 2b) based, in turn, on the "T-BAG" method

416   [160,161]. After SAM formation, divinylsulphone (DVS) was employed as a crosslinker

417   to attach aminated glycanes [162]. Phosphonate chemistry has demonstrated good

418   efficiency in the modification of a Silicon Nanowire-based DNA biosensor [163] and in

419   SAM formation on SiO_2 [164] or Ta_2O_5 [165]. This procedure is also the standard

420   surface modification protocol for the fluorescence microarray chips commercialized by

421   Zeptosens [166,167], made of Ta_2O_5 planar waveguides.

422   Other strategies involve the derivatization of the silicon or silicon nitride by

423   previously removing the native silicon oxide layer. Thus, porous silicon-based devices

424   have been derivatized by hydrosilylation by reacting Si-H bonds with alkene moieties

425   by thermal [168,169] or photochemical activation [170,171]. In this way, carboxylic

426   acid-ended surfaces are obtained and used for aminated probe attaching by EDC/NHS.

427   However, they are yet to be implemented in integrated optics due to long reaction times

428   and the special reaction conditions required. In any case, the employment of

429   photoinduced reactions, involving shorter times and better conditions, is an interesting

430   idea to develop alternative functionalizations for IO devices. Our research group has

431   derivatized silicon nitride with glutaraldehyde through surface N-H bonds after

432   removing the silicon oxide native layer (Figure 2c). This allows the selective attachment

433   of aminated probes against silicon oxide [172]. This approach was used for the

434   biofunctionalization of a highly sensitive silicon nitride slot waveguide microring

435   resonator [173], integrated with microfluidics, to perform excellent sensitivity [174].

436   Finally, other alternative approaches have also been explored by increasing probe

437   loading on the surface while minimizing non specific binding. They are based on the

438   use of dextran hydrogels or dendrimers. [175,176,177]. Thus Goddard *et al.* [175], after

439   a functionalization of a 1D PhC surface with APTMS, used EDC/NHS and a carboxy-

440   terminated dendrimer to attach aminated oligonucleotides (Figure 2f). By utilizing a

441 biotinylated dextran hydrogel, Vollmer *et al.* [177] attached streptavidin-conjugated

442 capture probes onto a silica microsphere cavity biosensor. Dextran-based

443 biofunctionalization has also been applied to IO devices developed on other materials,

444 such as Ta_2O_5 in a Waveguide Interrogated Optical Immunosensor (WIOS) [178].

445 The main biofunctionalization approaches employed to date on IO biosensors are

446 shown in Table S1 (Supplementary Material), and include reaction conditions, type of

447 capture agent and biorecognition event.

448 *2.1.3. Capture Agents Employed as Model Systems*

449 Having performed chemical functionalization on the surface, different capture agents

450 can be attached to the surface: biotin, proteins, antibodies, single-strand nucleic acids,

451 aptamers and carbohydrates.

452 This review classifies biosensors into two groups: those using a model system as

453 proof-of-concept devices and employing capture agents toward targets of analytical

454 interest, and even analyze real samples. The second group is discussed in more detail in

455 Section 3 (Main Achievements).

456 In proof-of-concept IO devices, two main model systems are used: a

457 biotin/streptavidin pair (BT-STV) and a bovine serum albumin/anti bovine serum

458 albumin pair (BSA/anti-BSA). BT-STV is used extensively thanks to its high affinity to

459 recognition and its capacity to display oriented bioreceptors, and it is a well-

460 documented bioaffinity system [53, 97, 100-103,110,111,114,116,118,150].

461 Biotin is used in silicon-based IO biosensors in two ways. First, biotin is the capture

462 agent used to detect streptavidin or avidin, and as a model system to demonstrate the

463 fabricated device's biosensing capability. Thus, avidin biosensing has been seen in IO

464 ring resonators [101,102,110,120], and streptavidin has been used as a target for the

465 biotinylated surfaces of microtoroid resonators [97,100,111], air slot silicon nitride

466 microdisk resonators [114], planar waveguides for Optical Waveguide Lightmode

467 Spectroscopy (OWLS) [103], Mach-Zehnder interferometers [109,150] and ring

468 resonators [116].

469 Second, a biotinylated surface is employed to immobilize a vast variety of biotin-

470 modified probe molecules according to the Avidin-Biotin Complexation (ABC)

471 technique [179,180]. Following this approach, biotinylated Concanavalin A has been

472 immobilized on silicon nitride sensing chips by Reflectometric Interference

473 Spectroscopy [112]. Biotinylated antibodies have also been attached to porous silicon

474 microcavity sensors [88,128], and biotinylated DNA has been employed as a capture

475 agent linked to the surface of a silica microsphere cavity sensor [177].

476 In other devices, streptavidin, covalently attached to the surface, has been used to

477 immobilize the biotinylated capture agent in an oriented fashion in order to perform real

478 sample biosensing [107,108,118,132] (Section 3).

479 The second model system is based on the BSA/anti-BSA (or HSA/anti-HSA) pair.

480 Many studies into biosensing performances have been reported with these systems

481 [84,86,87,102,106,173,174]. This is because BSA adsorbs very well onto the surface

482 and surface functionalization is not absolutely necessary (if surface modification is

483 done, no special care must be taken). BSA is adsorbed on SOI PhC [83,85] and is also

484 covalently immobilized merely to demonstrate protein detection [91], and no further

485 biorecognition event is performed. In other cases, BSA acts as a capture agent to

486 monitor the recognition of its specific antibody (anti-BSA), as in a bimodal waveguide

487 interferometer where BSA is adsorbed [84], and as a photonic crystal waveguide [106]

488 and a slot waveguide ring resonator [173], where BSA is covalently linked to the

489 surface.

490   Other uses of BSA have been presented in the literature. For instance, BSA has been

491   used to carry other moieties responsible for target recognition; e.g., sugars [82] or biotin

492   [117]. Similarly to the BSA/anti-BSA pair, human serum albumin (HSA) and its

493   specific antibody (anti-HSA) have been employed to demonstrate biosensing in silicon

494   nitride Mach-Zehnder interferometers [86,87] and in SOI microring resonators [101].

495   Another pair employed as a model, be it to a lesser extent, is the binding IgG/anti-IgG

496   pair where IgG antibodies are used as capture agents to recognize antiIgG

497   [63,88,109,125,128].

498   When the sensor's capability to detect hybridization events needs demonstrating,

499   oligonucleotide probes ca be covalently attached to the surface. Generally, a synthetic

500   fully complementary strand is used to perform hybridization event detection. Such

501   experiments have been carried out on porous silicon-based optical devices [89, 90, 171],

502   silicon nitride-based M-Z Interferometer [121] and SOI-based microring resonators

503   [137,141]. Apart from these, very few examples using receptors other than the

504   aforementioned ones are found in silicon-based IO devices [139,159].

505   After bioreceptor attachment, a blocking step is often performed to avoid non specific

506   binding, which can be achieved in two ways: using a blocking agent after bioreceptor

507   attachment. Thus, BSA has been widely used for this purpose, especially when proteins

508   or antibodies are employed as capture agents [88,107,113,134,135,136]. Commercial

509   blocking buffers like Starting Block [118,137,140,143] PEG [101,115,118] and

510   ovoalbumin protein (OVA) have also been employed. Generally in oligonucleotide

511   probes, no blocking step with protein is required, although the chemical blocking of the

512   remaining active sites is necessary. Thus, reducing agents [108] or ethanolamine

513   [58,89,172] are/is used to block aldehyde, isocyanate and epoxy-ended surfaces after

514   bioreceptor attachment. Second, designing surface functionalization chemistry helps

avoid biofouling, which is usually done with PEG derivatives [102,112,132] or dendrimers [177].

Sometimes, a blocking step is not necessary as non specific binding is negligible [105,133,137]. Non specific binding depends not only on surface modification, but also on the fluidics and buffers employed during biorecognition. Thus, the optimization of such variables can lead to a specific recognition of analytes on unblocked surfaces.

Comparing devices is difficult given lack of uniformity when optical biosensor sensitivity is given. So it is not infrequent that the target concentrations needed to distinguish the signal from noise background are very high, but much lower limits of detection from the interpolation in the saturation curve are reported. This is an important issue if the ultimate goal of the device is to act as a real sample biosensor. Table S2 (Supplementary Material) presents the sensitivities or limits of detection reported for the IO devices discussed herein, comprising both the lowest target concentration applied to the chip and the reported sensitivity data.

2.2. Techniques Employed for the Biofunctionalization of IO Devices.

From the biofunctionalization viewpoint, one important aspect is to place bioreceptors only in the device's sensing area so that sensitivity remains undiminished. For the time being, this has been accomplished by chemically selective reactions [98,99,172] or by covering the chip with a protecting layer with open windows, but only in the sensing area. This also allows the selective functionalization of each device sensor with the various specific receptors to perform multiplex detection.

The receptors are placed in the sensing zone by microspotting –either manually [102,105,133,137] or using a robotic spotter [82,109] – and by employing microfluidics [108,133,134,136,140,143,144]. Despite online functionalization being interesting in the initial stage when setting up and characterizing IO biosensors, it is not the best

540   option if chip fabrication is to be translated to a mass scale. This procedure is time-

541   consuming and presents other problems associated with the microfluidics, such as

542   leaking, channel blocking, cross contamination, etc.

543   *2.3. Characterization Techniques for Modified Surfaces*

544   To check the success of the functionalization process, different characterization

545   techniques are used. Figure 7 provides some examples of characterizations done on IO

546   biosensors. As an initial stage, many researchers use planar substrates to perform the

547   protocol. In general, characterization is accomplished in thickness by ellipsometry

548   [101,109,111,114,172], in hydrophobicity by contact angle measurements

549   [101,171,172], in chemical composition by XPS [98,101,111,115,159,172] and FT-IR

550   spectroscopy [90,92,172], and in topography by AFM [98,115]. Fluorescence

551   microscopy [92,101,114,172,177] and radio labeling [118,181] are also used to assess

552   the bioconjugation of the receptor of interest. However, fluorescence labeling is not

553   suitable when working with silicon surfaces due to this material's quenching effect on

554   tag emission [182]. This fact can be clearly observed in Figure 7a, where a microarray

555   (3x1) of Cy5-labeled goat anti-rabbit antibody (5 ppm in PBS1x) is printed on the

556   surface of Si, silicon nitride and SiO_2 chips respectively, and fluorescence is read with a

557   homemade surface fluorescence reader [183]. As seen, the fluorescence recorded for the

558   silicon surface is considerably lower than that for silicon oxide.

559   However, performances in planar sensors are not necessarily the same as on micro-

560   structured surfaces, and characterization techniques for nanostructures are also required.

561   In general, fluorescence microscopy (Figure 7b) [82,98-100,111,172], SEM (Figure 7c)

562   [97,99,113,172], and TOF-SIMs (Figure 7d) [159] have been used, but their

563   applications are scarce.

564   Another possibility is to use the IO device to monitor the whole functionalization

565   process step by step. For this purpose, microfluidics is used and success in the layer

566   formation is related with the device's RI response [58,104,133,137]. This is an

567   interesting option when the material does not allow the use of fluorescence microscopy

568   and techniques, like the SEM of TOF-SIMs, whose resolution is not sufficient to

569   provide useful information.

570   In conclusion, uniformity and rigor in the systematic characterization of the

571   biofunctionalization process are lacking. In many studies, detection of the target analyte

572   is considered sufficient and no further characterization is performed. In our opinion,

573   knowing the functionalization characteristics allows process modifications that help

574   improve the quality of intermediate layers, which will no doubt affect final biosensor

575   performance.

576

577   **3. Performances and Applications. Main Achievements**

578   This section briefly describes the most interesting advances in IO devices for real

579   sample biosensing. It is worth mentioning that almost all the significant developments

580   made involve targets of clinical interest.

581   The suitability of an optical biosensor for a particular application depends on its

582   performance over a variety of metrics. This includes technical aspects, such as

583   transducer sensitivity and bioassay sensitivity, as well as other parameters that evaluate

584   market success possibilities (ease-of-use, sensor cost, portability, scalability and

585   throughput).

586   Transducer sensitivity is defined as variation in either the sensor output response

587   resulting from a unit change refractive index (denominated bulk sensitivity) or the mass

588   density (called surface sensitivity) on the sensor surface. It is independent of a

biofunctionalization process being performed and is used for sensor characterization at a basic level. However, the bioassay sensitivity must be measured to evaluate the device's real biosensing capability and is the response variation to a given change in analyte concentration. It depends on both the biofunctionalization process and the affinity constant between receptor and analyte. Thus when working with the same optical device, high-molecular-weight molecules with a high affinity to the receptor are detected at lower concentrations than small molecules with a low binding affinity. It should be noted that the assay sensitivity value is mandatory if real biosensing is claimed.

Table 1 summarizes the most relevant results obtained to date in real sample biosensing using IO devices.

One platform demonstrating good performance for real sample biosensing is SOI microring resonators. Different assays have been performed in them using the hydrazine/aldehyde conjugation chemistry. By employing aminated DNA oligonucleotide probes attached to the surface, closely related bacterial species (*S. neumoniae and S. agalacticae*) are distinguished by monitoring specific probe hybridization with microRNAs (mRNAs) in a single, multiplexed assay; the smallest mRNA amount detected is 53 fmol [105]. This method has proven reproducible, and dehybridization with RNase enzyme enables sensor surface regeneration [133]. Sensitivity is improved by amplification using anti-DNA:RNA antibodies and by lowering the detected concentration to 350 amol (10 pM). With this device, the isothermal discrimination of DNA single nucleotide polymorphisms (SNPs) is achieved [137], and DNA-encoded antibodies against prostate specific antigen (PSA) and α-fetoprotein (AFP) are used to demonstrate the multiplex screening of capture agent binding properties [138,143]. The DNA encoding strategy consists in a self-assembly

process where antibodies are previously conjugated to specific sequences that are complementary to the DNA strands immobilized on the surface. It has an advantage over direct antibody covalent immobilization, that of improving its binding capacity (as it is oriented). Surface regeneration by a dehybridization step is also possible. Antibodies are also covalently attached to the surface to perform quantitative, multiplexed analyses of clinically relevant protein biomarkers: PSA, AFP, carcinoembryonic antigen (CEA), tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) by direct immunoassays [134,144]. Covalently linked antibodies are also used for the quantitation of biomarker C-reactive protein (CRP) in human serum and plasma directly and by a sandwich immunoassay, followed by a bead-enhanced third binding event to increase sensitivity [142]. Other interesting applications of such IO devices include: the specific, quantitative, multiplexed cytokine analysis of T-cell secretion by a one-step sandwich immunoassay [136,140], the multiplex binding kinetics determination of thrombin-binding DNA aptamer and anti-thrombin monoclonal antibody [139], or the quantitative detection of *Bean pod mottle virus* from leaf samples by direct immunoassay, involving quicker sample preparation and with a limit of detection of 10 ng mL^{-1} [135]. Carbohydrate-protein and norovirus particle interactions are characterized by the same microring resonator sensor array and organophosphonate based chemistry [159]. However, the results reported when this approach was adopted are preliminary, and the established and widely demonstrated methodology to functionalize microring resonators is that based on hydrazine-aldehyde conjugation.

Vertically coupled high refractive index microring resonators show the specific capture of whole bacteria cell *E. coli* O157:H7 by direct immunoassay after the covalent immobilization of the specific monoclonal antibody using GOPTS [113]. Although

control assays do not reveal the covalent nature of antibody attachment in this case, bacteria recognition proves specific.

By utilizing an integrated innovative bimodal waveguide interferometer, Duval *et al.* [58] recently demonstrated the picomolar detection level of human hormone hTSH by an indirect competitive immunoassay. In this case, carboxy-ended organosilane is used to functionalize the surface; while the hormone (antigen) is attached to the sensor through amine groups using EDC/NHS (no details of surface chemistry and characterization are provided). The specific antibody to hTSH is recognized by the receptor on the surface in an inversely proportional manner to the amount of hormone present in the sample. These methods are sensitive (20 pM), reproducible and specific.

With a Mach-Zehnder interferometer design with planar optical waveguides, Xu *et al.* [119] also specifically recognized three avian influenza virus subtypes by direct immunoassay, with limits of detection of $5 \cdot 10^{-4}$ hemagglutination units per milliliter (HA units mL^{-1}). In this case, SH-ended organosilane is used and specific antibodies are attached to the surface by a 1-hour incubation with m-Maleimidobenzoyl-N-hydroxysuccinimide ester as the crosslinker. Stability under storage at 4 ℃ for several days is demonstrated.

Ymeti *et al.* [56] used a four-channel integrated Young interferometer for the detection of herpes simplex virus type 1 (HSV-1) by a direct immunoassay with monoclonal specific antibodies immobilized on the surface via protein A in an oriented fashion. The whole process is developed online by monitoring real-time surface changes. Two points of this demonstrator must be highlighted; first, the specificity of the recognition is assessed by immobilizing different receptor layers in adjacent measuring channels and by monitoring the sensor response to different analyte solutions. Second, although protein A is adsorbed on the surface, negligible desorption

663 is noted for the analysis time, as observed from the baseline. Finally, solutions are

664 allowed to flow over the sensor for no longer than 30 min, and very good results are

665 obtained. Sensitivity is very high (850 particles mL^{-1}) and the blood serum analysis is

666 also reported, be it with lower sensitivity [81].

667 Antibodies covalently attached on the surface of a Hartman interferometer-based

668 optical chip have been reported by Schneider *et al.* [118] to detect human chorionic

669 gonadotropin (hCG) in serum and whole blood. For this purpose, a sandwich

670 immunoassay is performed using gold labeled secondary antibody to increase sensitivity

671 (0.1 ng mL^{-1}). Chemical surface functionalization is accomplished by silanization with

672 GOPTS, followed by oxidative epoxy ring opening to provide an aldehydized surface.

673 Then, avidin is attached to the surface by reductive amination and biotinylated-specific

674 antibodies are bound to this surface. However, the most interesting point of this work is

675 the study into non specific binding during measurements and how to compensate it

676 using a reference channel.

677 Qiao *et al.* [169] have demonstrated the first implementation of a porous silicon-based

678 Bloch surface sensor for protease activity detection (7 μg mL^{-1}) based on the digestion

679 of gelatin covalently attached to the surface. In this case, a hydrosilylation process is

680 employed as the first modification step. This approach comprises many further steps,

681 but sensitivity is poor; thus, it is not so interesting.

682 Armani *et al.* [184] have employed silicon-based microtoroids resonators to

683 demonstrate real biosensing with extremely low sensitivities. To that end, protein G is

684 adsorbed on the surface to bind by affinity-specific antibodies and to detect IL-2 in fetal

685 bovine serum with a sensitivity close to a single molecule ($5 \cdot 10^{-18}$ M). PhC-based

686 sensors have also demonstrated the capability of real biosensing. The so-called

687 nanoscale optofluidic sensor array (NOSA) [185] has been used for the multiplex

688   detection of three interleukins (IL-4, IL-6, IL-8) by sandwich immunoassays.

689   Streptavidin is covalently attached to the surface by using APTES and glutaraldehyde,

690   which allow the oriented immobilization of biotinylated capture antibodies. Despite

691   sensitivity not being that good (1 μg mL^{-1}), it is one of the first demonstrations of real

692   multiplex biosensing with PhC [108]. With the same device and by also employing

693   APTES and glutaraldehyde, four serotypes of Dengue virus were detected by

694   immobilizing four specific DNA oligonucleotides and performing hybridization after

695   adding the specific targets to the sensor [186].

696   As seen, the sensor biofunctionalization conditions employed are diverse and vary in

697   terms of assay times, blocking process, etc. To date, the most extensive, characterized

698   work is that developed with microring resonators using hydrazine-aldehyde chemistry.

699   A comparative study which adopts different approaches for the same sensor design and

700   for the same bioassay is still lacking, along with any advances made in this research

701   field.

702

703   **4. Future Trends**

704   One of the main advantages of IO technology is the possibility to integrate all

705   functions (chemistry, optics, fluidics and electronics) into a single platform. Despite

706   significant advances having been made in recent years toward developing IO biosensors

707   capable of acting as point-of-care or lab-on-a-chip devices, several issues remain to be

708   explored. The choice of the surface modification, biofunctionalization procedures, and

709   the detection assay type and conditions, become a relevant issue to consider, together

710   with the development and optimization of integrated optics-based sensing structures.

711   Some interesting aspects to be studied in the future, which have the potential to improve

712   already existing performance, are discussed in this section.

The effective and selective patterning for spatial control in the biofunctionalization of sensors to help accomplish high-density multiplexing has not yet been fully achieved. The use of automatic printers or microfluidic systems is a good option, but both techniques are quite time-consuming, and the search for easier and faster alternatives is an objective. In this sense, photoactivated coupling reactions, especially those in the click-chemistry group, have demonstrated their utility in planar platforms [146,187] and can be an interesting possibility to explore their application in the multiplex biofuncionalization of high-density array sensors.

Moreover, new conjugation techniques that simplify the number of steps and place capture agents close to the surface in a bioavailable manner, while providing a robust and reproducible link, are another unexploited field of study. The analyses of next-generation capture agents that improve the performance of those already in use are also desirable.

Designing approaches that allow surface regeneration and provide versatility, as in the DNA-encoding strategy, should be one of the preferred options for new developments. There is a large number of novelties in relation to capture agents for real biosensing to be explored in the IO devices whose efficiency has been demonstrated on other assay platforms, such as the triplex affinity capture methods or use of synthetic (aptamers) or semi-synthetic molecules (peptide nucleic acids, PNA) as new probes [188-191].

Another highly desirable option is to find biofunctionalization strategies that are compatible with the manufacturing process of devices. This would make them mass production scalable, and would help them reach truly applicable point-of-need devices ready for end users.

736   Finally, the establishment of a testing model system would facilitate the comparison

737   between devices. This system should include not only checking on specific binding, but

738   also stability tests of functionalized devices for long-term storage.

739   The issues discussed above rely only on the chemical functionalization of the surface

740   and anchoring of bioreceptors.

741   However, it is noteworthy that these developments have to be coupled with advances

742   in other fields such as optics, fluidics and electronics. It is only in this way that real

743   progress in the vast, multidisciplinary task of managing to manufacture RI integrated

744   optical devices capable of providing fast, specific and sensitive responses at the point of

745   need will be feasible.

746

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Figure Captions

Figure 1: Number of publications per year on the Refractive Index Optical BioSensing

topic during the last decade.

Figure 2: Scheme of the most relevant functionalization procedures on silicon-based

materials for IO biosensors: organosilane-based (a), phosphonate-based (b), and

glutaraldehyde-based (c) approaches.

Figure 3: Scheme of the bioconjugation procedures employed in IO biosensors for

aminated surfaces: N-hydroxysuccinimide-based (a), succinic anhydride-based (b), p-

phenylenediisocyanate-based (c), glutaraldehyde-based (d) hydrazine-aldehyde-based

(e) and carboxy-ended dendrimer-based (f) approaches.

Figure 4: Scheme of the bioconjugation procedures employed in IO biosensors for

thiol-ended surfaces: disulfide bridge-based (a), m-maleimidobenzoyl-N-

hydroxysuccinimide-based (b) and thiol-ended click chemistry-based (c) approaches.

Figure 5: Scheme of the bioconjugation procedures employed in IO biosensors for

epoxy-ended surfaces: nucleophilic ring opening-based (a), oxidative ring opening-

based (b) and photo-induced ring opening-based (c) approaches.

Figure 6: Scheme of the bioconjugation procedures for the carboxylated surfaces (a)

and isocyanate-ended surfaces (b), employed in IO biosensors.

Figure 7: Some examples of IO biosensors characterizations: a) Comparison between

intensity of fluorescence in a microarray of a Cy5-labeled antibody (same

concentration) deposited onto a silicon (left) and a silicon oxide (right) surface; b) SEM

images of the slot waveguides with streptavidin selectively attached on silicon nitride

following the chemical approach shown in Figure 2.c; c) Fluorescence confocal

microscopy characterization for the SiN slot waveguides modified according to the

procedure shown in Figure 2c, and using the Cy5-labeled antibody and 1% Fluorescein

isothiocyanate as a contrast; d) ToF-SIMS measurements (CN- and CNO- ions) on the waveguides functionalized with isocyanate-ended organosilane (red arrows show the defects on the organosilane layer formed on the surface), e) layout of an RI IO biosensor consisting of seven slot waveguide ring resonators, six sensing rings and a reference ring, a microscope image of a reference and a sensing ring (with an open window in the sensing area) and a measurement scheme.
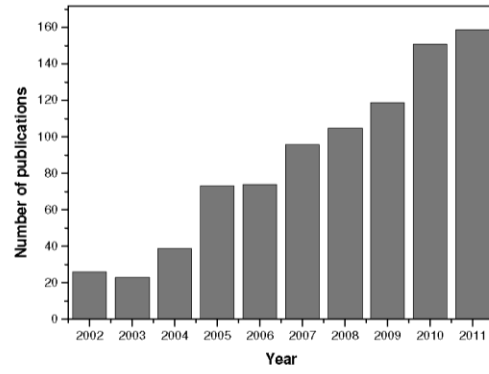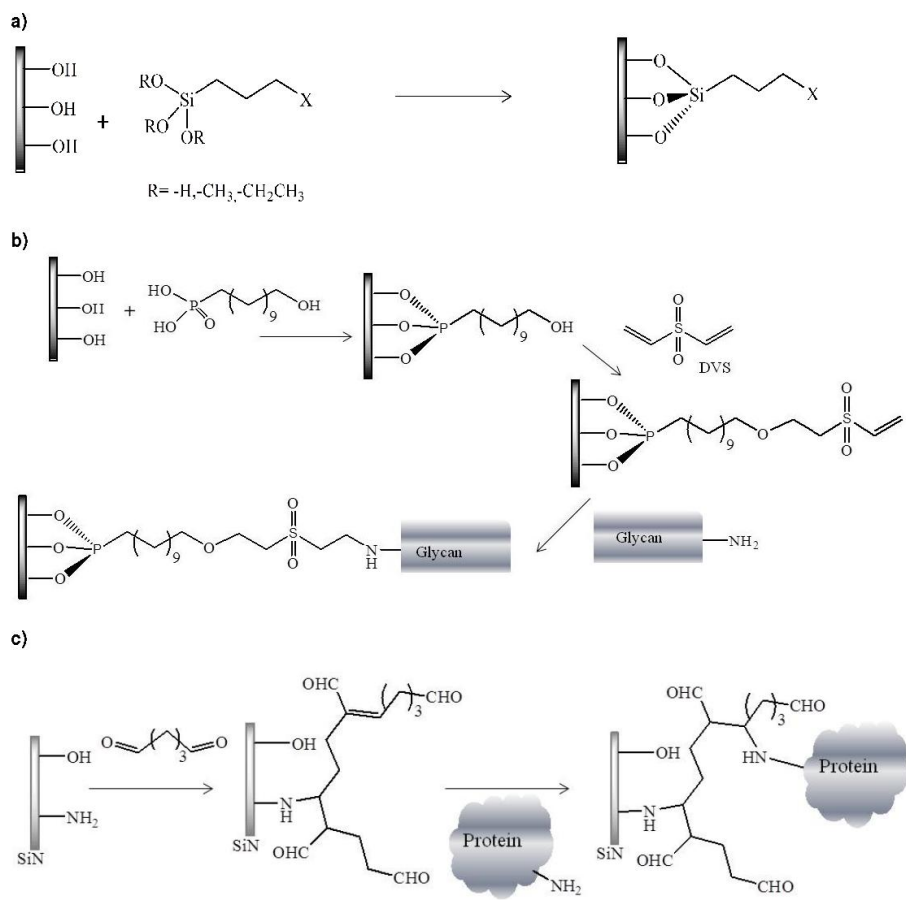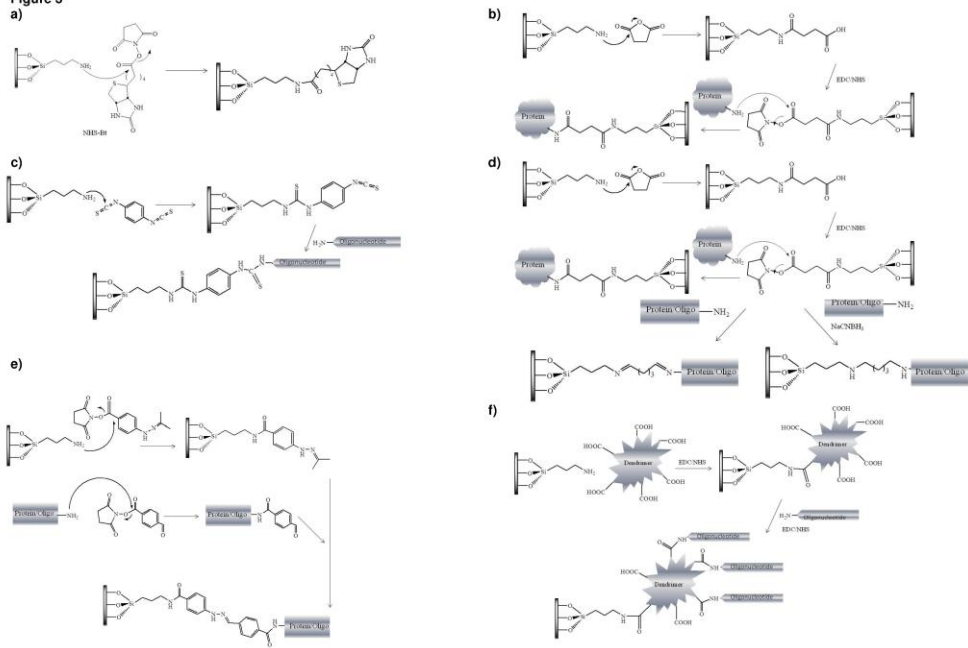
1143   **Figures:**

Figure 1

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Figure 2

a)

R= -H,-CH₃,-CH₂CH₃

b)

DVS

c)

Figure 3

a)

NHS-Bt

b)

EDC/NHS

Protein

c)

d)

EDC/NHS

Protein

Protein/Oligo — NH₂

NaCNBH₃

e)

f)

EDC/NHS

Dendrimer

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Figure 4

a)

b)

c)
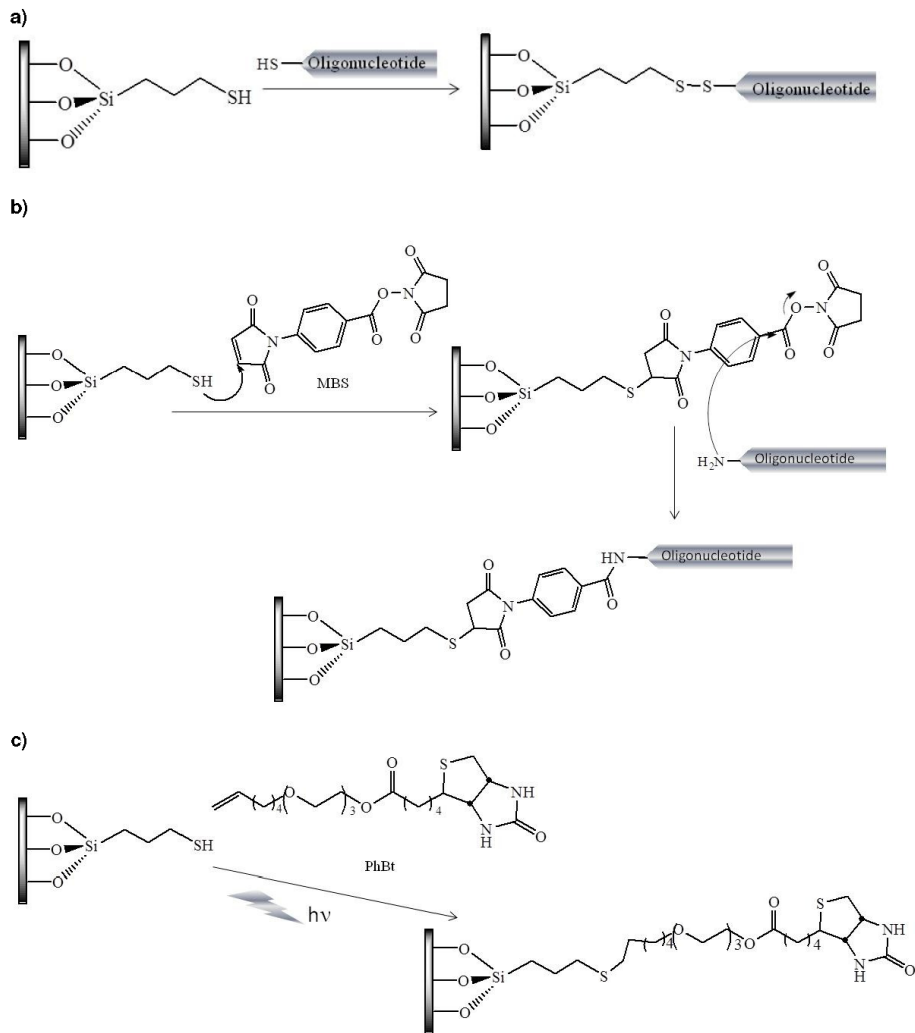
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Figure 5

a)

b)

c)

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Figure 6

a.1)

a.2)

b)

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Figure 7

a)

b)

c)

d)

e)