Immobilization, stabilization and patterning techniques for enzyme based sensor systems

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ABSTRACT

Sandia National Laboratories has recently opened the Chemical and Radiation Detection Laboratory (CRADL) in Livermore, CA to address the detection needs of a variety of government agencies (e.g., Department of Energy, Environmental Protection Agency, Department of Agriculture) as well as provide a fertile environment for the cooperative development of new industrial technologies. This laboratory consolidates a variety of existing chemical and radiation detection efforts and enables Sandia to expand into the novel area of biochemically based sensors. One aspect of our biosensor effort is further development and optimization of enzyme modified field effect transistors (EnFETs). Recent work has focused upon covalent attachment of enzymes to silicon dioxide and silicon nitride surfaces for EnFET fabrication. We are also investigating methods to pattern immobilized proteins; a critical component for development of array-based sensor systems. Novel enzyme stabilization procedures are key to patterning immobilized enzyme layers while maintaining enzyme activity. Results related to maximized enzyme loading, optimized enzyme activity and fluorescent imaging of patterned surfaces will be presented.

SUMMARY

Processing techniques for fabrication of improved enzyme modified field effect transistors (EnFET) are under development. To date, EnFET fabrication has been a two step process. Automated wafer scale microelectronic fabrication is followed by dicing and manual chip scale biochemical processing. The goals of the biochemical processing are attachment of enzyme to the exposed gate region of a FET and maintenance of enzyme catalytic activity. However, chip scale biochemical processing is tedious and difficult to reproduce. The processes under development emphasize wafer scale enzyme attachment, stabilization and patterning to facilitate device fabrication, enhance device uniformity and enable multi-enzyme processing. An overview of the process is presented in Fig. 1.

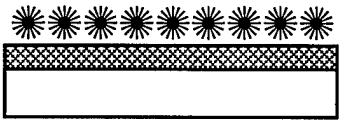
ENZYME IMMOBILIZATION

The enzyme acetylcholinesterase (AChE, Electrophorus electricus) was used in all tests. Activity of immobilized enzyme layers was measured by challenging with colorometric substrate (propionylthiocholine). The immobilization strategy used was adapted directly from Weetall and coworkers.[1] Amine derivatized surfaces are generated by treating the substrate with an aminosilane then the bifunctional linker glutaraldehyde is used to connect the surface amines to free amines on the enzyme. Glutaraldehyde treatment and enzyme contact are performed sequentially to minimize enzyme crosslinking. The utility of this chemistry with silicon dioxide or silicon nitride films has been demonstrated previously.[2] Aqueous silanization with a trifunctional silane (aminopropyltriethoxysilane, APTS) was selected. Aqueous silanization precludes the use and disposal of organic solvents, a significant concern for wafer scale processing. The immobilization procedure of Bhatia et al.[3] (mercaptopropyltrimethoxy-silane[MTS] followed by gamma-maleimidobutyrloxy succinimide ester[GMBS]) was also tested since this process was used previously specifically for AChE immobilization. The two chemistries gave equivalent enzyme surface activities, though the MTS/GMBS does result in more uniform surface activity. (Fig 2) However, the mercaptosilane derivitization is carried out in dry toluene in an inert atmosphere of nitrogen. Therefore, it is not readily adapted to wafer handling systems and has significant waste disposal costs. A wide variety of enzyme immobilization strategies have been investigated[4] and the APTS-glutaraldehyde is by no means the only process compatible with wafer scale processing. Avoiding specialized reaction conditions (low temperature requirements of cyanogen bromide reactions) or equipment (nitrogen glove box) facilitates microelectronic and biochemical process integration.

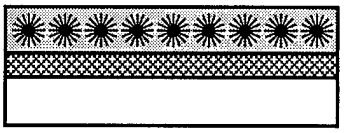
DISCLAIMER

Initial Structure:
Si Substrate with
100nm SiO_2

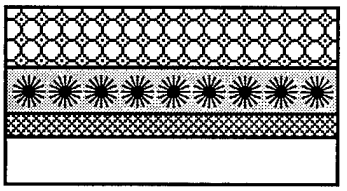
Process:
Attach enzyme with
aminosilane and
glutaraldehyde



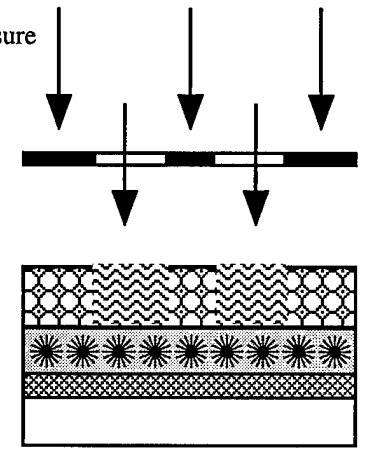
Process:
Soak in stabilizer
Spin dry, dehydrate



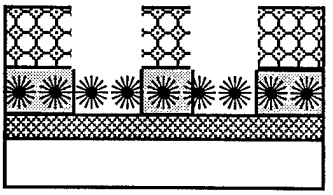
Process:
Spin coat with
positive photoresist



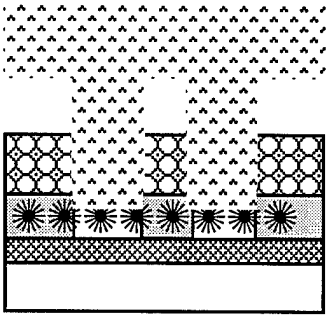
Process:
Selective UV exposure
with mask aligner



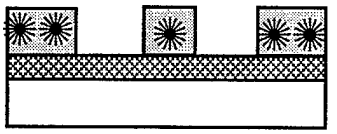
Process:
Develop exposedresist
Rinse removes stabilizer



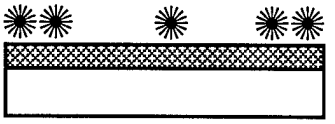
Process:
Inactivate/etch enzyme
with O_2 plasma



Process:
Acetone strip photoresist



Process:
Rinse removes stabilizer
Must keep surface wet or
retreat with stabilizer



Process:
Challenge with fluorescent
tag. Inspect with
fluorescent microscope

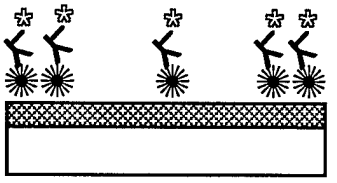


Figure 1: Overview of enzyme patterning via photolithography and oxygen plasma exposure

ENZYME STABILIZATION AND PHOTORESIST PROCESSING

The essence of the process presented is patterning immobilized enzyme layers in the same fashion used to pattern inorganic thin films by the microelectronics industry - microphotolithography followed by selective etching. Previous work demonstrated successful use of this approach to pattern immobilized antibody layers.[5] The successful integration of biochemical components with photoresist processing is dependent upon defining stabilization conditions and/or protection layers which enable the sensitive biocomponents to withstand the chemical, temperature, and radiation treatments associated with resist processing. Treatment of immobilized enzymes with cyclic polyalcohol sugars has been shown to enable enzyme dehydration without loss of activity.[6] After testing several sugars unsuccessfully, a commercially available enzyme linked immunoassay (ELISA) stabilization product was found suitable. Formulations are proprietary but typically contain a mixture of sugar and protein. Immobilized AChE enzyme layers are soaked in stabilization solution then dehydrated in a vacuum oven. Standard positive photoresist (OCG 825) was then applied but with modified bake temperature and time due to enzyme temperature sensitivity. Fig. 3 presents immobilized enzyme activity of the same wafer after each significant process step. The wafer was reprocessed through all previous processes before each additional process step. The stabilization solution enables immobilized enzyme to withstand dehydration and coating with photoresist. There is activity loss after resist coating but this is possibly due to desorption of non-specifically bound enzyme. There was much less loss of enzyme activity after the second coating with photoresist. Enzyme layers not soaked in stabilization solution displayed no activity after dehydration or resist coating.

ENZYME INACTIVATION / ETCHING

After resist development, numerous wet chemical strategies are feasible to inactivate the exposed enzymes, e.g., crosslinking or acid digestion. However, these techniques must be compatible with the resist layer and result in additional liquid chemical waste. An oxygen plasma provides a clean, low temperature ambient in which neutral gas species react with surface materials and form volatile products (etching). Therefore, the plasma not only inactivates exposed proteins but oxidizes and removes them so that the substrate is suitable for immobilization of additional enzyme layers. The ability of this technique to etch immobilized antibody layers has been reported previously and was confirmed with ellipsometric surface analysis[6]. Enzyme layers not stabilized and protected with photoresist display no activity after oxygen plasma treatment. Oxygen plasmas are routinely used to etch photoresist from wafers after patterning steps. However, this lack of etch rate selectivity is not a concern. Patterned resist can still serve as an etch mask when patterning biological components because of the vast difference in thickness between the photoresist etch mask (1-2 um) and the protein layer being etched (5 - 15 nm in this immobilized enzyme case).

CONCLUSION

The process summarized in Fig. 1 and the preliminary results presented here combined with previous success patterning immobilized antibody layers demonstrate a novel procedure for immobilized enzyme patterning. This process is completely analagous to and compatible with standard microelectronics thin film processing. Preparation of fluorescently tagged antibody specific for the immobilized enzyme is currently underway. Challenge of patterned enzyme surfaces with this labeled antibody will enable visualization of the patterned enzyme layers. Future work will focus upon reiteration of the process to pattern additional enzyme layers on the same substrate enabling preparation of multiple EnFETs on the same chip.

REFERENCES

1. Weetall, H. H., (1976), in *Methods in Enzymology*, vol 44, Mosbach, K., ed., Academic, NY, 134-148.

2. Flounders, A. W., Brandon, D. L., Bates, A. H. (1995). Immobilization of thiabendazole-specific monoclonal antibodies to silicon substrates via aqueous silanization. *Applied Biochem. Biotechnol.* **50**(3) 265.

3. Bhatia, S. K., Cooney, M. J., Shriver-Lake, L. C., Fare, T. L., Ligler, F. S. (1991). Immobilization of acetylcholinesterase on solid surfaces: chemistry and activity studies. *Sensors and Actuators B*, **3**, 311.

4. Weetall, H. H. (1993). Preparation of immobilized proteins covalently coupled through silane coupling agents to inorganic supports. *Appl. Biochem. Biotechnol.* **41**(3), 157.

5. Flounders, A. W., Brandon, D. L., Bates, A. H. Patterning of Immobilized Antibody Layers via Photolithography and Oxygen Plasma Exposure. *Biosensors & Bioelectronics* Accepted Ocober 1996.

6. Gibson, T. D. and Woodward, J. R., (1992). Protein stabilization in biosensor systems. In: Edelman, P. G. and Wang, J., (eds.) *Biosensors and Chemical Sensors*, ACS Symposium Series 487, Washington, D.C.

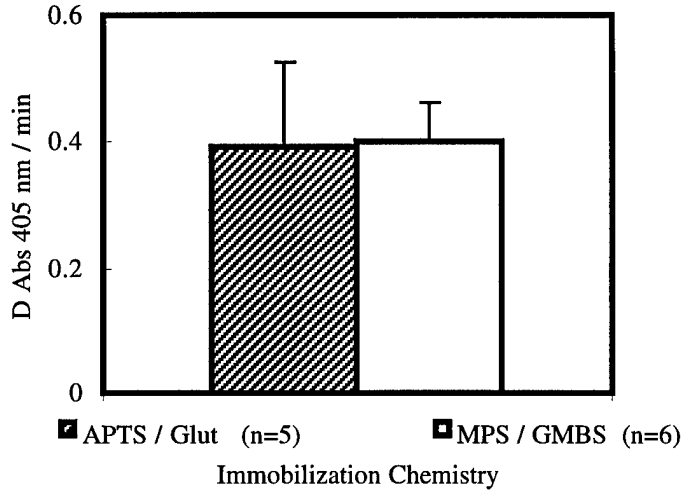


Figure 2: Immobilized enzyme activity of two different attachment chemistries.

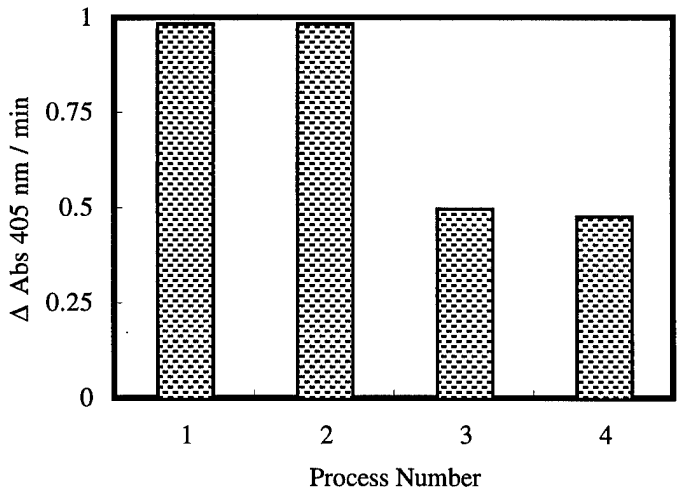


Figure 3: Immobilized enzyme activity after wafer processing
Process 1: After immobilization
Process 2: After 1 + soak in stabilizer and dehydration
Process 3: After 1+2+ spin coating with photoresist
Process 4: After 1+2+3+ oxygen plasma exposure

Report Number (14) SAND--97-8496C
CONF-970231--

Publ. Date (11) ~~DCTER~~ 199701

Sponsor Code (18) DOE/ER, XF

JC Category (19) UC-406, DOE/ER

DOE