Summary
The thesis deals with the activation of inert polymer materials and covalent immobilization of biomolecules onto such polymers by novel unconventional method. We have activated polycarbonate and polystyrene matrix and used them for preparation of immobilized biosystem for different applications. The present technique permits covalent binding of biomolecules to the solid support under gentle reaction conditions. Versatile applications of immobilized biomolecules are another important aspect of the thesis.

The polycarbonate matrix coated with FNAB was efficiently activated by irradiating UV light of 365 nm in 12 minutes. The optimum concentration of FNAB for activation of area of 0.3 cm^2 on both side of the polycarbonate strip was found to be 15 μmol which corresponds to 2.73 mg/strip. The activated surface possess labile fluoro group which can easily be displaced by any nucleophilic reagent and allows covalent binding with the matrix. The activated polycarbonate strip was used for covalent immobilization of ascorbate oxidase. The optimum temperature and time of immobilization were found to be 50°C and 45 min, respectively. The immobilized-AOX onto polycarbonate strips shows higher pH and thermal stability over that of the free enzyme. Immobilized-AOX has improved storage life and can be stored for longer duration. The immobilized-AOX polycarbonate strips were successfully used for rapid detection of L-ascorbic acid in fruit juice samples. As immobilization of ascorbate oxidase was carried out on plastic surface, the procedure could be useful in developing low cost disposable strip system for rapid analysis of ascorbic acid from the sample.

The photochemical activation of polycarbonate matrix and covalent ligation of protein molecules were characterized by X-ray photoelectron (XP) spectroscopy. XP spectra of activated polycarbonate matrix distinctly showed the presence of fluorine and nitrogen peaks in addition to the carbon and oxygen peaks, present in the untreated matrix. XP spectra of activated polycarbonate matrix after protein immobilization showed disappearance of the fluorine peak, which clearly indicates that binding occurs through the covalent linkage by displacement of fluoro group by the amino group of the protein.
In chapter three, we activated the surface of polystyrene microtiter wells in a photochemical reaction by using the photolinker, FNAB to carry out immobilization. Pressure energy was used to carry out the protein immobilization onto the activated surface instead of conventional thermal energy. The optimum amount of FNAB was found to be 10 μmol for activation of a well of a polystyrene microtiter plate and the time of UV exposure was 12 minutes. The optimum pressure and time required for covalent binding of Horseradish peroxidase to the activated surface was 2.0x10^5 Pa and 25 min, respectively. Thus, the pressure reduced the temporal constraints for covalent ligation of protein to the activated surface by 5 fold, compared to conventional method. Horseradish peroxidase immobilized by pressure showed better thermal and storage stability with respect to free enzyme. Reusability study shows that immobilized-enzyme retained its original activity after three repetitive uses; however activity falls to half after ten-cycles of repeated use. The Michaelis–Menten constant (Km) and maximum reaction velocity (Vmax) for pressure-induced immobilized HRP onto the activated surface were found to be 55.3 μM and 0.957 mM/min, respectively, while, for free enzyme the corresponding values were 34.7 μM and 1.089 mM/min. The method was found to be equally effective for immobilization of other enzymes including glucose oxidase, alkaline phosphatase and invertase.

Thus, the method of protein immobilization by pressure is simple, rapid and efficient, and can be used for immobilization of biomolecules onto activated surface by manipulating the pressure energy and duration of applied pressure. Besides, the method can be used to immobilize proteins on any surface provided the surface is active enough to facilitate covalent linkage. Further, no heating is required during immobilization by pressure; hence the method could be a potential alternative for conventional thermal incubation for immobilization of biomolecules, in particular for heat sensitive biomolecules where temperature is a limiting factor, and useful for various applications including immunoassay, preparation of biosensors, biochips and microarrays.

Thereafter, we showed the application and effectiveness of pressure energy over thermal energy via well-known technique, ELISA by replacing conventional thermal incubations with pressure. The optimum conditions of pressure and time of applied pressure for maximum antigen immobilization was 1.5 x10^5 Pa in 10 minutes.
Further, we also performed rest of the ELISA steps by pressure and optimized the conditions. The ELISA procedure carried out under optimized conditions took only an hour, without involving any costly monoclonal antibody or sensitive tag. Pressure-mediated ELISA carried out in 1 h gives similar ELISA value to that of conventional and heat-mediated ELISA methods and also gives more than three-fold increase in ELISA value than the control experiment carried out in same time and temperature without applying pressure. The estimation of total human IgE by 1 h PELISA method gives ELISA value akin to that obtained by 3 h-HELISA method. Since PELISA method proved rapid, sensitive, specific and reproducible (intra- and inter assay CVs were less than 10%) for the studied proteins, it can be an excellent alternative to conventional ELISA method, widely used method in clinical and research laboratories.

At last, but not least, we exploited the surface activation chemistry of FNAB for fabrication of oligonucleotide probes to the photoactivated matrix in a simple and single-step method. The oligonucleotide probe was covalently ligated to the activated polystyrene surface by displacing the labile fluoro group of the activated surface with the amino group of the modified oligonucleotide probe. Maximum immobilization of oligonucleotide probe is achieved in one hour at 60°C. Further, it was checked that the ligation of the oligonucleotide probes to the activated surface is due to the amine modification. Optimized time for hybridization of target oligonucleotide to with immobilized probe onto the solid surface was 2 h at 45°C. The method is equally good for immobilizing different oligonucleotides and does not depend on the base composition of the sequence. Covalently immobilized probe showed high selectivity in subsequent hybridization processes with the complementary target and clearly discriminates single-base mismatch target from the complementary target. Thus, the method is a useful protocol for the detection of single nucleotide polymorphism. The reported method for covalent immobilization of oligonucleotides and its hybridization was a qualitative assay. Nevertheless, quantitative measurement can be achieved by incorporating a fluorescent tag; thereby enhancing the applicability of this method further in diagnostics and microarray techniques. The method is one of the shortest routes for immobilization of oligonucleotide probes to the polymer support. The method is versatile, as it can immobilize amine- or thiol-modified oligonucleotide onto FNAB-activated variety of polymer surfaces.
In conclusion, we showed a simple method for covalent immobilization of ascorbate oxidase onto polycarbonate strip for ascorbic acid detection. A novel rapid and unconventional method of protein immobilization technique using pressure as the energy source is also developed. This technique does not require conventional thermal incubation; hence useful for immobilization of thermolabile biomolecules. Another interesting aspect of the thesis is pressure-mediated-ELISA (PELISA). First time we have shown that pressure, a mechanical force, covalently immobilizes a biomolecule without affecting its biological properties. We have also demonstrated that pressure enhances non-covalent interaction of biomolecules, such as antigen-antibody binding. In last chapter, we have developed a one-step method for covalent immobilization of oligonucleotide probes onto activated surface which is potentially useful for designing DNA based diagnostics, biochips, biosensors and microarray technology in a much reduced cost.
Bibliography


Chen, S., Shih, J.C., Xu, Q.P., 1985. 4-Fluoro-3-nitrophenyl azide, a selective photoaffinity label for type B monoamine oxidase. Biochem. Pharmacol. 34, 781-788.


Bibliography


for improved immobilization of proteins by the epoxy method. Biomacromolecules 4, 772-777.


Appendix
APPENDIX

(A) Materials

Ascorbate oxidase (AOX), horseradish peroxidase (HRP), bovine serum albumin (BSA), invertase, goat anti-human IgG, human IgG, rabbit IgG, goat anti-human IgG-peroxidase conjugate, streptavidin-peroxidase, 4-fluoro-3-nitroaniline, sodium dodecyl sulfate (SDS), D-glucose, o-phenylenediamine dihydrochloride (OPD) and p-nitrophenylphosphate (pNPP) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Glucose oxidase (GOD) and alkaline phosphatase (AP) were purchased from Boehringer-Mannheim (Germany) and Sigma-Aldrich, St. Louis, MO, USA. Human IgE ELISA kit was purchased from Bethyl Laboratories, USA.

Ascorbic acid, 2,6-dichloroindophenol, m-phosphoric acid, ethanol, methanol, carbon tetrachloride, hydrogen peroxide, sodium nitrite, sodium azide, sodium chloride, sodium phosphate monobasic, sodium phosphate diabasic, sodium hydrogen carbonate, sodium carbonate, tween-20, o-dianisidine and other chemicals were of analytical grade and purchased either from S.D. Fine Chemical, India, Himedia, India, Central drug house, India, Merck, India, Sisco research laboratories Pvt. Ltd. India or Thomas Baker, India. Polystyrene microtiter plates were purchased from Greiner Bio-One, Frickenhausen, Germany and polycarbonate sheet was purchased locally.

(B) Reagents

Synthetic oligonucleotide sequences were purchased from Midland Certified Reagent Company, Texas, USA, shown in Table 5.1.

Table 1. Oligonucleotide probes and targets

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Oligonucleotide Sequence</th>
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<tbody>
<tr>
<td>Probe-0</td>
<td>5’-Biotin–ACA AGA CGT TTT ACA GTT GC-3’–NH₂</td>
</tr>
<tr>
<td>Probe-00</td>
<td>5’-Biotin–ACA AGA CGT TTT ACA GTT GC-3’</td>
</tr>
<tr>
<td>Probe-1</td>
<td>5’-NH₂–ACA AGA CGT TTT ACA GTT GC-3’</td>
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<tr>
<td>Probe-2</td>
<td>5’-NH₂–ATG TGG AAA ATC TCT AGC AG-3’</td>
</tr>
<tr>
<td>Probe-3</td>
<td>5’-NH₂–TCG GGG TTT TGG GTC TGA CG-3’</td>
</tr>
<tr>
<td>Oligonucleotide</td>
<td>Oligonucleotide Sequence</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------------------------------------------</td>
</tr>
<tr>
<td>Target-1</td>
<td>5’-Biotin-GCA ACT GTA AAA CGT CTT GT-3’</td>
</tr>
<tr>
<td>Target-2</td>
<td>5’-Biotin-CTG CTA GAG ATT TTC CAC AT-3’</td>
</tr>
<tr>
<td>Target-3</td>
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<tr>
<td>Mismatch-2</td>
<td>5’-Biotin-GCA GCT GTA CAA CGA CTT GT-3’</td>
</tr>
</tbody>
</table>

(C) Buffers

**Carbonate/bicarbonate buffer (0.1 M, pH 9.6)**

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<th>Component</th>
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<tr>
<td>Na₂CO₃</td>
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<tr>
<td>NaHCO₃</td>
<td>5.04 g</td>
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<tr>
<td>H₂O</td>
<td>up to 1000 mL</td>
</tr>
</tbody>
</table>

pH adjusted to 9.6 ± 0.2.

**Phosphate Buffer (0.01 M, pH 7.2)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>0.35 g</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>1.34 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>up to 1000 mL</td>
</tr>
</tbody>
</table>

pH adjusted to 7.2 ± 0.2.

**Phosphate Buffer Saline (0.01 M, 0.15 M NaCl, pH 7.2)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>0.35 g</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>1.34 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.47 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>up to 1000 mL</td>
</tr>
</tbody>
</table>

pH adjusted to 7.2 ± 0.2.

**Washing Buffer (0.01 M PB, 0.15 M NaCl, pH 7.2, and 0.1% Tween-20)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>0.35 g</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>1.34 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.47 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>up to 1000 mL (pH adjusted to 7.2 ± 0.2)</td>
</tr>
<tr>
<td>Tween-20</td>
<td>1 mL</td>
</tr>
</tbody>
</table>
Phosphate Buffer Saline for Oligonucleotide Study (0.1 M, 1 M NaCl, pH 7.2)

NaH₂PO₄·H₂O - 3.792 g
Na₂HPO₄·2H₂O - 12.175 g
NaCl - 58.5 g
H₂O - upto 1000 mL
pH adjusted to 7.2 ± 0.2

Washing Buffer for Oligonucleotide Study (0.1 M, 1 M NaCl, pH 7.2 and 0.1% SDS)

NaH₂PO₄·H₂O - 3.792 g
Na₂HPO₄·2H₂O - 12.175 g
NaCl - 58.5 g
H₂O - upto 1000 mL (pH adjusted to 7.2 ± 0.2)
SDS - 1 g

Citrate Buffer (0.2 M Citric acid-phosphate buffer, pH 5.0)

Citric acid - 0.526 g
Na₂HPO₄·2H₂O - 1.014 g
H₂O - upto 100 mL
pH adjusted to 5.0 ± 0.2

Substrate dye reagent for HRP

o-Phenylenediamine - 4 mg
H₂O₂ (30%) - 4 μL
Citrate buffer - upto 12 mL

Stop Solution (5% Sulphuric Acid)

H₂SO₄ - 5 mL
H₂O - 95 mL