Chapter 4

Immobilization of whole cells on biocompatible materials: Use as Enzyme Source for the Biotransformation of Arachidonic acid to 19-HETE and 20-HETE
4.1. Summary

A variety of materials can be utilized for immobilization of *Candida bombicola* based on different immobilization protocols. These biomaterials can be used as enzyme sources for the transformation of arachidonic acid to vasoactive compounds 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE). In general few points need to be considered before choosing the materials for the whole cell immobilization.

1. The materials should be stable, robust and inert. It must be biocompatible and should not interfere with bioreaction.
2. The materials should protect the whole cell containing reactive enzyme against microbial deterioration and render the enzyme accessible to cofactor, metal ions etc.
3. The materials must permit substrate accessibility to immobilized whole cells and thus avoid mass transfer problem.
4. The materials should have high immobilization whole cell loading factor for the efficient transformation reaction.
5. The immobilization process must be simple, quick, inexpensive and ecofriendly.

The studies in this chapter are focused on immobilization of *Candida bombicola* on novel biocompatible supports as an enzyme sources for the transformation of arachidonic acid to bioactive molecules, 19-hydroxyeicosatetraenoic acid (19-HETE) and 20- hydroxyeicosatetraenoic acid (20-HETE). This chapter is divided into two subchapters for three different biocompatible materials used for immobilization of *Candida bombicola*. 
4.2. Introduction

Impressive advances are being made in the synthesis of chemically functionalized and patterned biocompatible surfaces for the immobilization of biomolecules such as whole cells and enzymes of microorganisms such as bacteria and yeast.\textsuperscript{1, 2} Development of methodologies for the entrapment and immobilization of whole cells has important implications in a range of applications, examples of which include basic cell biology,\textsuperscript{3, 4} biosensing,\textsuperscript{5} tissue engineering\textsuperscript{6} and treatment of diseases by controlled delivery of biological products.\textsuperscript{7} One of the challenges in this area is to develop protocols wherein the immobilization of the cells is to be spatially controlled, preferably on a submicron to micron scale by designing surfaces of varying “adsorptivity” of the biological system. This gives an insight into the effect of cell shape and cell function\textsuperscript{3} which enhances our ability to control the cellular environment and helps in understanding fundamental cell biology. Such patterned surfaces for immobilization of cells have been obtained using microcontact printing (\(\mu\)-CP) on reactive\textsuperscript{3} and mixed self-assembled monolayers (SAMs).\textsuperscript{8} SAMs of alkanethiolates\textsuperscript{9} and alkylsilanes\textsuperscript{10} were obtained by the sol-gel technique\textsuperscript{11} and using elastomeric membranes.\textsuperscript{12} Groves et. al., in 2001 have demonstrated that phospholipid bilayers act as biomimetic surfaces and modulate the assembly and growth of cells.\textsuperscript{13} Recently \(\mu\)-CP of organic monolayers and subsequent polymer functionalization has been used to develop patterns in the seeding of bacterial cells.\textsuperscript{14}

An important application of bacterial and fungal cells (genetically engineered and otherwise) is use as “factories” for the production of industrially and medically important enzymes and metabolites.\textsuperscript{15} Here we have been interested in assembly of specific cells on
surfaces from the point of view of using the cells as sources of enzymes for biotransformations and synthesis of new materials. The enzyme cytochrome P450 present in the yeast cells was used to catalyze in situ ω and ω -1 hydroxylation of arachidonic acid. Cytochrome P450, the enzyme of interest is unstable outside the cellular environment and in such cases, immobilization of the whole cells was important to catalyze reactions that are dependent on the unstable enzymes. As part of our search for newer and more versatile materials, tailorable surfaces for cell immobilization has been used. This chapter presents the synthesis of different biocompatible materials whose surface may readily be modified to render it compatible for a variety of biocatalytic applications.
Chapter 4. A

*Candida bombicola* cells immobilized on patterned lipid films as enzyme sources for the transformation of arachidonic acid to 19-HETE and 20-HETE

Chapter 4. B

Nanogold membrane as scaffolds for whole cell immobilization as enzyme source for biotransformations of arachidonic acid to 19-HETE and 20-HETE
Chapter 4. A: Candida bombicola cells immobilized on patterned lipid films as enzyme sources for the transformation of arachidonic acid to 19-HETE and 20-HETE

4.A.1. Introduction

Chapter 4.A. part presents the assembly of Candida bombicola yeast cells onto patterned thermally evaporated fatty amine thin films (octadecylamine, ODA) and the use of the enzyme cytochrome P450 present in the yeast cells to catalyze in-situ the ω-hydroxylation of arachidonic acid (AA) to 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE)\(^ {16, 17}\) (see Figure 4.A.1.). Cytochrome P450 is a membrane bound protein and is known to be highly unstable outside the cells.\(^ {17}\) Thus, rather than immobilizing the unstable purified enzyme within a lipid film, we demonstrate here the immobilization of Candida bombicola yeast cells carrying the enzyme cytochrome P450.\(^ {16, 17}\) This immobilization affords a cheaper, renewable and more versatile alternative for carrying out the transformation of arachidonic acid to 19-HETE and 20-HETE. In view of the vasoactive and renal pharmacological activity of 19-HETE and 20-HETE,\(^ {18}\) the large-scale production of this molecule is of commercial interest and the methodology presented herein assumes added importance.

Part of the work presented in this chapter 4.A. has been published in: Biotechnol. Prog. 2003, 19, 1659-1663.
Figure 4.A.1. Transformation of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) and 19-hydroxyeicosatetraenoic acid (19-HETE) mediated by cytochrome P450 enzyme present in Candida bombicola cells.

The immobilization of the Candida bombicola cells was accomplished by simple immersion of the patterned ODA film into an aqueous dispersion of the cells as illustrated in Scheme 4.A.1. The assembly of the cells on the ODA surface occurs possibly through hydrophobic interactions between the cell wall and the ODA molecules (Scheme 4.A.1.) and thus provides a rational strategy for assembly of the cells. The adhesion of the cells to the ODA surface is sufficiently strong and permits reuse of the immobilized cells in the biochemical transformation of arachidonic acid to 20-HETE.
Scheme 4.A.1. Illustration of immobilization of *Candida bombicola* yeast cells on thermally evaporated ODA film surface
4.A.2. Materials and Methods

4.A.2.1. Chemicals

Arachidonic acid of 99% purity and octadecylamine (ODA) of 97% purity were obtained from Sigma and Aldrich Chemicals respectively and used as received. All reagents were from standard commercial sources and of highest quality available.

4.A.2.2. Growth of Candida bombicola cells

Candida bombicola cells ATCC 22214 were pre-cultivated in 50 mL medium at 30°C consisting of (g l⁻¹): glucose, 100; yeast extract, 1; (NH₄)₂SO₄, 1; MgSO₄ · 7H₂O, 0.3; Na₂HPO₄, 2; NaH₂PO₄, 7; pH 5.5 and shaken at 160 rpm. Cells from the late growth phase (24 h) were inoculated into 1 lit. flasks containing 400 mL medium. Fermentative procedure employed for the experiment was same as described previously in Chapter 2 section (2.2.3.).

4.A.2.3. Deposition of octadecylamine films

250 Å thick films of ODA (CH₃-(CH₂)₁₇–NH₂) were thermally evaporated onto 6 MHz AT-cut quartz crystals for quartz crystal microgravimetry (QCM) measurements, Si (111) wafers (for Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) measurements) in an Edwards E308 vacuum coating unit. A 40 μm x 40 μm mesh size transmission electron microscope (TEM) grid was used as a mask in the deposition of patterned ODA films on the Si (111) substrate. The deposition was done at a pressure of 1 x 10⁻⁷ Torr and the film deposition rate and thickness were monitored in-situ using an Edwards quartz crystal thickness monitor.
4.A.2.4. **Immobilization of Candida bombicola cells onto the thermally evaporated ODA films**

The immobilization of the yeast cells on the ODA surface was followed by QCM by immersion of the 250 Å thick ODA film on gold-coated AT cut quartz crystals for different time intervals in an aqueous dispersion of the cells (~ $10^8$ cells/mL) and measuring the frequency change of the crystals *ex-situ* after thorough washing and drying of the crystals. The frequency counter used was an Edwards FTM5 instrument operating at a frequency stability and resolution of ± 1 Hz. For a 6 MHz crystal used in the investigation, this translates into a mass resolution of 12 ng/cm$^2$. The frequency change was converted to mass loading using the Sauerbrey formula.\(^{19}\) The 250 Å thick ODA films on Si (111) substrates were immersed in the *Candida bombicola* cells dispersed in deionized water for 4 h. This optimum time of immersion was estimated from QCM measurements. For the reusability of the cells immobilized on 250 Å thick ODA films surface, were washed 3 times by deionized water prior to reuse.

4.A.2.5. **FTIR measurements**

FTIR measurements of the 250 Å thick ODA films deposited on Si (111) substrates before and after immobilization of the *Candida bombicola* cells were made on a Perkin Elmer Spectrum-1 FTIR spectrometer operated in the diffuse reflectance mode at a resolution of $4\text{ cm}^{-1}$. 
4.A.2.6. Scanning electron microscopy (SEM)

The immobilization of the *Candida bombicola* cells on the patterned ODA films was also studied by scanning electron microscopy (SEM) on a Leica Stereoscan-440 electron microscope. Presence of cells on the patterned ODA film surface after incubation of the cells immobilized on ODA surface after 96 h in the reaction medium was confirmed from SEM images.

4.A.2.7. Synthesis of sophorolipids

The yeast cells immobilized on the 250 Å thick ODA films were immersed in the reaction medium containing 5 mL 10% sterile glucose and 30 mg arachidonic acid and was incubated for 96 h at 30°C under slow shaking. After the reaction, the supernatant was decanted and used for extracting the sophorolipid.17


Acid hydrolysis of the sophorolipids under N2 with 1M HCl for 12 h at 25°C liberated the fatty acids, which were extracted with an equal volume of chloroform. Hydroxylated fatty acids were purified on 500 mg Aminopropyl Sep-Pak Cartridges (Waters). Samples in 0.5 mL chloroform were applied to cartridge pre-equilibrated with 5 mL n-hexane. Neutral lipids were eluted with 25 mL chloroform /2-propanol (2:1, v/v), mono-hydroxylated fatty acids with 25 mL 2% (v/v) acetic acid in diethyl ether and phospholipids with 25 mL methanol. The hydroxy fatty acid fraction was rotary evaporated and the residue taken up in a small volume of chloroform. Hydroxy fatty acids were purified by TLC on standard Kiesel-gel 60 plates and developed with petroleum ether (b.p. 60-80°C) diethyl ether/ acetic acid (50:50:1, by volume). Iodine
vapors were used to visualize fatty acids and the corresponding bands were immediately eluted with methanol/chloroform (2:1 v/v) and derivatized to their methyl ester silyl ethers.\textsuperscript{17, 20}

4.A.2.9. Gas chromatography mass spectroscopy (GC-MS)

A Shimadzu GCMS QP 5050 automated quadrupole mass spectrometer operating in the electron impact mode. GC parameters: column used, BP-5 fused silica column (30m x 0.25 mm, 0.25mm, 0.25mm coatings). He gas as a carrier at 14 kPa head pressure; injector at 250 °C; column initially at 150 °C for 1 min (rate 35 °C/min) then increased to 220 °C for 5 min (rate 5 °C/min) and then at 280 °C and then held at these conditions for 10 min; injection volume 1 µL. MS parameters: Interface temperature 250 °C, ionization mode electron impact, scan range 70 to 450 amu (arbitrary mass units) s\textsuperscript{-1}. 
4.A.3. Results and discussions

4.A.3.1. QCM studies

The kinetics of cell immobilization onto the thermally evaporated ODA films was monitored by immersion of a 250 Å thick ODA covered QCM crystal for different time intervals in the cells dispersed in deionized water and monitoring the change in resonance frequency of the crystal ex-situ after thorough washing and drying of the crystals. Since the mass of the individual cells is not known, we have used the frequency change alone as an indicator of the attachment of the cells on the hydrophobic ODA surface. Figure 4.A.2. (A) has shown the QCM mass uptake data recorded during immobilization of Candida bombicola cells to the ODA film surface. The error bars are based on an analysis of three separate QCM measurements. It is observed from the Figure 4.A.2. (A) that there is a rapid attachment of the cells initially with 90 % of the cells being immobilized within the first 100 minutes of immersion. The cell density on the ODA film surface eventually reaches saturation after 4 h of immersion in the yeast cell suspension. In all further experiments, this optimum time of immersion (4 h) in the Candida bombicola cells solution was used to obtain films of the immobilized cells.
Figure 4.A.2. (A) QCM mass uptake data recorded during immobilization of *Candida bombicola* cells onto a 250 Å thick ODA film deposited on an AT-cut 6 MHz quartz crystal as a function of time of immersion in the cell suspension. The error bars indicate 10 % deviation to the data from their mean values as determined from the three separate measurements.
4.A.3.2. FTIR Studies

FTIR spectroscopy provides a convenient means of monitoring the attachment of the Candida bombicola cells via fingerprint signatures of cellular components. Figure 4.A.2. (B) has shown the FTIR spectra recorded from a 250 Å thick as-deposited ODA film (curve 1) and the ODA film after immersion in Candida bombicola cells solution for 4 h (curve 2). Two prominent features labeled a (917 cm\(^{-1}\)) and b (1110 cm\(^{-1}\)) in the figure can be seen for the cell-immobilized ODA film (curve 2) which are clearly absent in the as-deposited ODA film (curve 1). These two absorption bands a and b are characteristic of excitation of deoxyribose-phosphate vibration modes and vibrations in the deoxyribose groups in the DNA molecules of the yeast cells respectively.\(^{21}\) The FTIR results thus present additional evidence for the presence of the Candida bombicola cells on the ODA film surface.
Figure 4.A.2. (B) FTIR spectra recorded from an as-deposited 250 Å thick ODA film (curve 1) and the ODA film after complete immobilization of the *Candida bombicola* cells (curve 2) on Si (111) substrates.
4.A.3.3. SEM measurements

Figure 4.A.3. (A) has shown the SEM image of the as-deposited ODA film on a Si (111) substrate using a TEM grid as a mask. It is seen that well-defined individual hexagonal elements of the ODA film have been deposited on the substrate. Figure 4.A.3. (B) and (C) have shown energy dispersive analysis of x-rays (EDAX) spot profile analysis on masked (marked as in Figure 4.A.3. (A)) and exposed substrate (marked as + in Figure 4.A.3. (A)) of the patterned surfaces of ODA lipid films. Figure 4.A.3. (C) has shown nitrogen signal from the exposed surface, hence confirms the deposition of the ODA, however nitrogen signals were absent from the masked region, this confirms that ODA is not deposited in this region. Thereafter, this film was immersed in an aqueous suspension of Candida bombicola cells for 4 h and washed thoroughly prior to imaging by SEM.

Figures 4.A.4. (A) and (B) have shown the low and high magnification SEM image recorded after immobilization of the Candida bombicola cells onto the hexagonal ODA patterns. It is clearly seen from SEM image that the yeast cells are immobilized extremely faithfully on the ODA elements with negligible binding of the cells to the exposed silicon surface.
Figure 4.A.3. (A) SEM images recorded from patterned thermally evaporated ODA thin film. (B) and (C) shows EDAX spot profile analysis on masked. [In Figure 4.A.3. (A) marked as $x$ and exposed surface of patterned ODA lipid films (marked as $+$)]

Figures 4.A.4 (A) and (B). low and high magnification of SEM images after immobilization of *Candida bombicola* cells onto the ODA film surface.
4.A.3.4. Synthesis of sophorolipids

As mentioned briefly in the introduction, our interest in Candida bombicola cells centres on the ability of the cells to catalyse the transformation of arachidonic acid to 20-HETE (Figure 1) and therefore, it is of paramount importance to establish the viability of the immobilized cells in performing this biochemical function. Films of the immobilized cells were reacted with arachidonic acid and the sophorolipids were isolated from the reaction medium and subjected to acid hydrolysis as described in detail in the experimental section. The immobilized yeast cells transformed 75% of the arachidonic acid to sophorolipid, which was then subjected to acid hydrolysis to yield 20-HETE. The overall reaction leading to the formation of 20-HETE from arachidonic acid was shown in Figure 4.A.1.

4.A.3.5. Acid hydrolysis of sophorolipids and isolation of 19-HETE and 20-HETE

As mentioned briefly in the introduction, our interest in Candida bombicola cells centers on the ability of the cells to catalyze the transformation of arachidonic acid to 19-HETE and 20-HETE. The sophorolipids formed during the biotransformations of arachidonic acid were subjected to acid hydrolysis to yield 19-HETE and 20-HETE compounds. The hydroxyecosatetraenoic acids were reacted with diazomethane solution and thereafter with the bis silyl trimethyl fluroacetamide (BSTFA) to give methyl ester silyl ether of hydroxyecosatetraenoic acid.

Figure 4.A.5. has shown the partial mass spectrum of 20-HETE as detected by GC-MS. Significant ions occurred at m/z [406 M⁺], 391 [M⁺ - 15], 316 [M⁺ - 90], 304 [(M⁺ +1) – 103]. Selective ion monitoring also showed a prominent signal at m/z 103 [(CH₃)₃-Si-O⁺-CH₂]. The mass spectrum thus clearly indicates that the hydroxyl group...
is at the C20 position and that the compound was 20-hydroxy 5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE). Confirmation of the identity of 20-HETE was obtained by the observed co-elution of 20-HETE standard by GC-MS with the isolated material.

The mass spectrum indicated a hydroxyl group at position C19. The mass spectrum of ions occurred at m/z 117 due to cleavage from C19 to C20; [(CH₃)₃-Si- O⁺-CH-CH₃] and 73 [base ion; (CH₃)₃-Si] and loss of 220 [M⁺ - 186] due to rearrangement and loss of –CH=CH (CH₂)₃-CH[O⁺-Si-(CH₃)₃]-CH₃, 201 {M⁺ - [131 + 74 (silyl group + H)]}. Figure 4.A.6. has shown the structures of the significant ions occurred of methyl ester silyl ether of 19-HETE. The compound was identified as 19-hydroxyeicosatetraenoic acid. Also yeast cells such as Candida apicola have been shown to synthesize sophorolipids in which sophorose, as a diglucoside is linked glycosidically to the terminal (n-) or sub terminal (n-1) hydroxy l group of a hydroxyl fatty acid. We would like to add that the film of the immobilized cells could be reused after reaction and thorough washing with only a marginal loss in activity (5 % cells over 5 reuse cycles) indicating that the cells were strongly bound to the underlying ODA film surface and that there is little leaching out of the cells during reaction. This is confirmed by imaging the ODA patterned surface after incubating the cells in the reaction medium for 96 h. the presence of the cells on the ODA film surface after reaction.
Figure 4.A.5. Partial electron impact mass spectrum of 20-HETE as detected by GC-MS. The compounds were extracted from sophorolipids, synthesized by Candida bombicola cells and was identified as the methyl ethyl silyl ether of 20-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE)
Figure 4.A.6. Partial electron impact mass spectrum of 19-HETE as detected by GC-MS. The compounds were extracted from sophorolipids, synthesized by *Candida bombicola* cells and was identified as the methyl ethyl silyl ether of 19-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (19-HETE)
Figure 4.A.7. (A) and (B) have shown SEM images of different regions of the Candida bombicola cells bound to ODA lipid films after one cycles of reaction (incubating in the reaction medium for 30 °C for 96 h). Thus, the yeast cells were strongly bound to the hydrophobic ODA lipid film permitting excellent reuse. The films of the immobilized cells could be reused after reaction and through washing with only a marginal loss in biocatalytic activity ca. 10 % after 5 cycles, indicating that the cells are strongly bound to the ODA lipid films.

Figure 4.A.7. Low (A) and high (B) magnification SEM images of Candida bombicola whole cells immobilized on thermally evaporated octadecylamine lipid films after one cycle of reaction.
4.A.4. Conclusion

In this study, we have demonstrated the immobilization of *Candida bombicola* yeast cells on patterned thermally evaporated ODA films, the assembly of cells to ODA films possibly driven by hydrophobic interactions between cell walls and ODA molecules. The immobilized yeast cells were biologically active and cytochrome P450 enzyme present in the *Candida bombicola* cells could be used to transform arachidonic acid to 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE). The biocomposite films are easily separated from the reaction medium for additional reuse.
Chapter 4. B: Nanogold membrane as scaffolds for whole cell immobilization as enzyme source for biotransformations of arachidonic acid to 19-HETE and 20-HETE

4.B.1. Introduction

Chapter 4.B. focuses on the preparation of chemically functionalized biocompatible polymeric membrane embedded by the gold nanoparticles and hydrophobized by using octadecylamine (ODA). The free standing hydrophobic nanogold membrane provides a biocompatible surface for the immobilization of whole cells. The attachment of the cells to the ODA bound to the nanogold membrane occurs possibly through the nonspecific interactions such as hydrophobic interactions between the cell walls and the ODA molecules. The enzyme, cytochrome P450 present in the immobilized yeast cells on the ODA film surface was used for the transformation of the arachidonic acid (AA) to sophorolipids and thereafter sophorolipids were acid hydrolyzed to liberate 19- hydroxyeicosatetraneoic acid (19-HETE) and 20-hydroxyeicosatetraneoic acid (20-HETE). The advantage of using whole cells is that it limits us to use cofactors such as NADPH for the synthesis of sophorolipids.

The synthesis of a free-standing gold nanoparticle membrane at the interface between chloroform containing bis (2-(4-aminophenoxy)ethyl)ether (DAEE) and aqueous chloroauric acid solution.

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Thereafter the nanogold membrane is hydrophobized by binding the octadecylamine (ODA) molecules to the gold nanoparticles embedded on the polymeric membrane and were used for the immobilization of whole cells.

The membrane is formed spontaneously by the reduction of AuCl₄⁻ ions by DAEE at the liquid-liquid interface, this process leading to the formation of gold nanoparticles. The concomitant process of oxidation of DAEE leads to the creation of a polymeric matrix in which the gold nanoparticles were embedded (Figure 4.B.1, step 1). The gold nanoparticle membrane was extremely stable, robust, easily handled, malleable and can be grown over large areas and thickness by suitably varying the experimental conditions. Hydroxylation was done by simple immersion of the nanogold membrane in octadecylamine (ODA) in ethanol solution for 12 h resulted in binding of ODA molecules to the gold nanoparticles through the amine groups. The immobilization of the Candida bombicola cells was accomplished by simple immersion of the hydrophobic nanogold membrane into an aqueous dispersion of the cells (Figure 4.B.1, step 2). The assembly of the cells on the hydrophobic nanogold membrane surface occurred possibly through hydrophobic interactions between the cell wall and the ODA molecules (Figure 4.B.1, step 2) and thus provides a rational strategy for assembly of the cells. The adhesion of the cells to the ODA surface is sufficiently strong and permits reuse of the immobilized cells in the biochemical transformation of arachidonic acid to 20-HETE (Scheme 4.B.1.). Moreover, the free standing nanogold membrane could be easily separated from the reaction medium and were reused.
Scheme 4.B.1. Transformation of Arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) mediated by cytochrome P450 enzyme present in *Candida bombicola* yeast cells.
Figure 4.B.1. Illustration of hydrophobization of nanogold membrane using octadecylamine and thereafter, immobilization of the *Candida bombicola* whole cells on the hydrophobic nanogold membrane

Hydrophobic nanogold poly membrane
4. B. 2. Materials and Methods

4. B. 2.1. Chemicals

Arachidonic acid (99 % purity), ODA (CH$_3$-(CH$_2$)$_{17}$-NH$_2$) were obtained from Sigma and Aldrich Chemicals respectively and used as-received. Ethyl alcohol (99.5%) was obtained from E-Merck, Germany. Bis (2-(4-aminophenoxy)ethyl)ether (DAEE) was prepared as: A mixture of potassium 4-nitrophenoxide (3.0 g, 16.9 mmol), 2-chloroethyl ether (1.21 g, 8.4 mmol) and ethylene glycol (15 mL) was stirred at 130 °C for 3 h. The cooled mixture was poured into ice-cold water. The pale yellow solid separated was filtered and recrystallized from ethanol. Yield 60 %, m. p. 155-156 °C. (Lit.1 154-157 °C)

Bis (2-(4-nitrophenoxy) ethyl) ether (0.8 g, 2.28 mmol) 10 mL of ethanol and 1 mg of 5 % Pd /C catalyst were placed in a flask fitted with a reflux condenser. Hydrazine hydrate (64 %, 2 mL) was added to the reaction mixture over a period of 10 min at room temperature and then the reaction mixture was refluxed for 16 h. The hot mixture was filtered and cooled. The solvent was stripped off to yield a waxy solid. Yield 92 % m. p. 59-61 °C. (Lit.2 59-60 °C)

4. B. 2.2. Growth of Candida bombicola cells

Fermentative procedure employed for the experiment was same as described previously in Chapter 2 section (2.2.3.)

4. B. 2.3. Nanogold membrane synthesis

In a typical experiment, 100 mL of $10^{-2}$ M concentrated aqueous solution of chloroauric acid (HAuCl$_4$) was mixed with 100 mL of $10^{-2}$ M DAEE in chloroform for 30 min. The membrane formed at the interface was separated and repeatedly washed with deionized water and were used for enzyme immobilization. The amount of gold
nanoparticles in the membrane was determined by atomic absorption spectroscopy (AAS). 10 mg of nanogold membrane was dissolved in 20 mL freshly prepared saturated I₂ solution in KI and volume was made up to 100 mL using deionized water. The solution was analyzed by a Varian Spectra AA 220 atomic absorption spectrometer (AAS) and was compared with the standard of gold solution to estimate the weight percent of gold nanoparticles in the membrane. The gold nanoparticles leached from the polymeric membrane were also used for the immobilization of whole cells.

4.B.2.4. Hydrophobization of nanogold membrane

10 mg of the nanogold membrane was dispersed in 10⁻² M ODA solution prepared in ethyl alcohol for 12 h. The nanogold membranes were then washed with copious amount of alcohol and chloroform and dried in the air for further use.

4.B.2.5. UV-Vis spectroscopy studies

UV-visible spectra of gold nanoparticles embedded in the polymeric membrane were recorded on a quartz substrate using a Jasco V570 UV/VIS/NIR spectrophotometer operated at a resolution of 1 nm. The probable structure of the nanogold membrane is illustrated in Figure 4.B.1.

4.B.2.6. Transmission Electron Microscopy (TEM) measurements

TEM measurements were performed on a JEOL Model 1200EX instrument operated at an accelerating voltage of 120 kV. Samples for TEM analysis were prepared by transferring a nanogold membrane from the liquid-liquid interface on carbon-coated TEM copper grids. The mixtures were allowed to dry for 1 min following which the extra solution was removed using a blotting paper. TEM measurements of gold nanoparticles leached from the nanogold membranes were also recorded.
4.B.2.7. FTIR measurements

FTIR measurements of the nanogold membrane formed on Si (111) substrates before and after binding of octadecylamine (ODA) were made on a Perkin Elmer Spectrum-1 FTIR spectrometer operated in the diffuse reflectance mode at a resolution of 4 cm\(^{-1}\).

4.B.2.8. Scanning electron microscopy (SEM) and energy dispersive analysis of X-rays (EDAX) measurements

The immobilization of the *Candida bombicola* cells on the as prepared and hydrophobic nanogold membrane were studied by scanning electron microscopy (SEM) on a Leica Stereoscan-440 electron microscope. Spot-profile energy dispersive analysis of X-rays (EDAX) measurements were performed to test the faithfulness of cell immobilization onto the surface of the nanogold membranes using a Phoenix EDAX attachment connected to the scanning electron microscope. Presence of cells on the hydrophobic nanogold membrane surface after incubation of the cells after 96 h in the reaction medium was confirmed by SEM images. SEM images of the gold nanoparticles leached from the polymeric membrane before and after immobilization of cells were also recorded.

4.B.2.9. X-ray diffraction measurement (XRD)

XRD measurements of gold nanoparticles bound to the polymeric membrane were done on a Philips PW 1830 instrument operating at 40 kV and a current of 30 mA with Cu K\(_\alpha\) radiation.
4.B.2.10. Immobilization of Candida bombicola cells onto the hydrophobic nanogold membrane

20 mg of hydrophobic nanogold membranes were then immersed in an aqueous dispersion of the cells (~ $10^8$ cells/mL) for 4 h (the optimum time of immersion was estimated from our earlier experiments). The amount of cells immobilized on the hydrophobic nanogold membrane was estimated from the initial and the final cell counts after immobilization. To determine the confidence limit, separate measurements were made for 3 different hydrophobic nanogold membranes. For the reusability of the cells immobilized on surface of hydrophobic nanogold membrane, it was washed 3 times by deionized water prior to reuse. Nanogold membrane and gold nanoparticles leached polymeric membrane were also used for the immobilization of the whole cells.

4.B.2.11. Synthesis of sophorolipids

The yeast cells immobilized on the 20 mg of hydrophobic nanogold membrane were immersed in the reaction medium containing 5 mL 10% of sterile glucose and 30 mg arachidonic acid in 200 µl alcohol and was incubated for 96 h at 30 °C under slow shaking. After the reaction, the supernatant was decanted and used for extracting sophorolipid.


Acid hydrolysis of the sophorolipids under N₂ with 1M HCl for 12 h at 25 °C liberated the fatty acids, which were extracted with an equal volume of in chloroform. Hydroxylated fatty acids were purified as described in section 4.A.2.8. of chapter 4.
4.B.2.13. Gas chromatography mass spectroscopy (GC-MS)

A Shimadzu GC-MS QP 5050 automated quadrupole mass spectrometer operating in the electron impact mode was used as described in section 4.A.2.9. of chapter 4.

4.B.3. Results and discussions

4.B.3.1. Preparation of the nanogold membrane material

Figure 4.B.2. (A) has shown the UV-Vis spectrum recorded in the transmission mode from the thin film of nanogold membrane transferred onto a quartz substrate. A strong absorption band centered at 540 nm is observed. This absorption was due to excitation of surface plasmons in gold nanoparticles and is responsible for their vivid pink-purple colour. The amine groups of DAEE molecules at the interface were protonated (pH of HAuCl₄ solution ~ 3.2) leading to electrostatic complexation with AuCl₄⁻ ions. That the electrostatic complexation with gold ions was a crucial step in the formation of the gold nanoparticle membrane was indicated by the control experiment where a similar interfacial reaction was carried out with the aqueous HAuCl₄ solution maintained at pH 9. At this pH, the amine groups in DAEE would not be protonated and no membrane formation was observed even after 12 h of reaction. Reduction of chloroaurate ions takes place at the interface and the oxidized DAEE molecules cap the spontaneously formed gold nanoparticles preventing their further aggregation. The nanogold membrane could be formed either by simple cross-linking of the gold nanoparticles through the terminal groups of oxidized DAEE or through formation of a polymeric network of the oxidized DAEE molecules. The estimation of gold nanoparticles in polymeric membrane was done by leaching the gold nanoparticles using
a saturated I$_2$ solution in KI and solutions were analyzed by atomic absorption spectrometry (AAS) as described in the experimental section. The nanogold membrane was kept in the iodine solution for more than 5 hours. The mass loading of the gold nanoparticles in the polymeric membrane was estimated as 30 weight %.

Figure 4.B.2. (A) UV-vis spectra recorded from the as-prepared nanogold membrane on quartz substrate
4.B.3.2. FTIR studies

Figure 4.B.2. (B) has shown the FTIR spectra recorded from a nanogold membrane before (curve 1) and after (curve 2) hydrophobization with the ODA molecules by immersion in $10^{-2}$ M ODA solution formed in absolute alcohol for 12 h. Two prominent features labeled $a$ (2850 cm$^{-1}$) and $b$ (2920 cm$^{-1}$) were due to the methylene antisymmetric and symmetric vibrations from the hydrocarbon chains of octadecylamine molecules bound to the nanogold membranes which were clearly absent in the as prepared nanogold membranes (curve 1). The frequency of these resonance indicated the ODA molecules on the gold particle surface were in closed-packed state. The hydrophobization of gold nanoparticles using octadecylamine molecules and the binding of the ODA molecules to the nanogold membranes occured through amine groups.$^{24, 25}$

Figure 4.B.2. (B) FTIR spectra recorded from the nanogold membrane before (curve 1) and after (curve 2) hydrophobization with the octadecylamine (ODA).
4.B.3.3. XRD and EDAX measurements

Figure 4.B.3. has shown the powder XRD pattern of the nanogold membrane. The Bragg reflections in the nanogold membrane clearly correspond to presence of gold. The presence of intense (311) reflection in the XRD pattern suggested oriented growth of the gold nanoparticles in the polymeric membrane along these crystallographic planes. This confirmed the reduction of chloroaurate ions at the liquid-liquid interface for the formation of gold nanoparticles. Spot profile EDAX measurements were done on the nanogold polymeric membrane. The prominent Au signal confirms the fidelity of gold nanoparticles in the polymeric membrane. However, the chlorine signals were also seen which were attributed to the unreduced gold ions (AuCl$_4^-$) were present in the membrane, presumably bound to the surface of the gold nanoparticles.

![XRD pattern and EDAX profile](image)

Figure 4.B.3. XRD patterns recorded from the gold nanoparticle membrane. Inset shows the spot profile EDAX recorded form the gold nanoparticle polymeric membrane.
4.B.3.4. TEM measurements

Figure 4.B.4 (A) and (B) have shown the low and high magnification of TEM micrographs respectively. It was seen that the gold nanoparticles were fairly dispersed in the polymeric membrane with little extent of aggregation in the membrane. As mentioned previously the chloroaurate ions formed electrostatic complex with the protonated amine groups of DAEE molecules and then are reduced. After reducing the chloroaurate ions the oxidized DAEE molecules formed a polymer and oxidized DAEE molecules cap the spontaneously formed gold nanoparticles preventing their further aggregation. The gold ions were electrostatically bound to protonated DAEE molecules and were highly localized at the liquid-liquid interface. Reduction of the gold ions by DAEE must clearly lead to oxidation of DAEE molecules in a highly localized manner. DAEE molecules possessed two terminal aniline segments, which were known to be good reducing agents. Oxidation of DAEE most probably proceeded through formation of a polymeric network derived from DAEE at the liquid-liquid interface. The gold atoms formed by the reduction of AuCl₄⁻ ions diffused along the polymeric network, aggregate into larger gold nanoparticles as seen in the Figure 4.B.4 (A) and (B), thereby yielding a polymeric network with inclusions of gold nanoparticles at the liquid-liquid interface. As shown in Figures 4.B.4. (C) and (D) the gold nanoparticles leached from the nanogold membranes by iodine treatment as described in the 4.B.2.6 section. The dark spots seen in Figures 4.B.4. (A) and (B) corresponding to the gold nanoparticles were not seen in the gold nanoparticles leached membrane. However, the dark regions in Figures 4.B.4. (C) and (D) corresponded to the entrapped iodine molecules during the leaching the gold nanoparticles. This was confirmed from the diffraction measurements.
Figure 4.B.4. (A) and (B) Low and high magnification of TEM micrographs of the free standing nanogold membrane.

Figures 4.B.4. (C) and (D) The TEM micrographs of the gold nanoparticles leached from the nanogold membrane.
4.B.3.5. SEM

Figure 4.B.5. (A) and (B) have shown the low and high magnification of SEM images of the nanogold membrane transferred from the liquid-liquid interface on the Si (111) substrates. It was seen that surface of the nanogold membranes was not smooth. Moreover, a mesh like structures were seen to form which indicated the simple cross-linking of the gold nanoparticles through the terminal groups of oxidized DAEE or through formation of a polymeric network of the oxidized DAEE molecules. As mentioned briefly earlier, the first step in the formation of the membrane was electrostatic complexation of AuCl$_4^-$ ions with protonated amine groups of DAEE molecules at the liquid-liquid interface and incomplete reduction of the gold ions would explain their presence in the membrane.

Figure 4.B.5. (A) and (B) Low and high magnification of SEM images of nanogold membrane synthesized at the liquid-liquid interface and transferred on Si(111) substrate.

Thereafter, this nanogold membrane was immersed in an aqueous suspension of Candida bombicola cells for 4 h and washed thoroughly prior to imaging by SEM.

Figure 4.B.6. (A) and (B) have shown the SEM images recorded after immobilization of
the *Candida bombicola* cells on to the hydrophobic nanogold membrane. It was clearly seen from the SEM images that yeast cells were immobilized extremely faithfully on the surface of the hydrophobic nanogold membranes. It was well known that these *Candida bombicola* cells bound to hydrophobic regions.\(^{23}\) The process of attachment of the cells to the hydrophobic nanogold membranes has been illustrated in Figure 4.B.1. The low and high magnification of SEM images recorded after immobilization of the *Candida bombicola* cells on to the as prepared nanogold membrane as shown in Figure 4.B.6. (C) and (D). The density of the cells was less as compared to the cells immobilized on the hydrophobic nanogold membranes. It was well known that the cells bind to hydrophobic regions of the surface\(^ {23}\) and thus the use of octadecylamine bound to the nanogold membranes provided the hydrophobic surface for the binding of the *Candida bombicola* yeast cells. The process of the attachment of the *Candida bombicola* yeast cells to the hydrophobic nanogold membrane has been illustrated in Figure 4.B.1. In order to understand the interactions between the polymer membrane and the *Candida bombicola* yeast cells, the gold nanoparticles leached polymeric membrane was used for the immobilization of whole cells. Care was taken to wash the polymeric membranes with copious amount of chloroform and after with deionized water prior to use.
Figure 4.B.6. (A) and (B) The SEM images of the *Candida bombicola* cell immobilized on the hydrophobic nanogold membranes.

Figure 4.B.6. (C) and (D). The *Candida bombicola* cells immobilized on the as prepared nanogold membrane.
Figure 4.B.7. (A) and (B) have shown the SEM images after immersion of the gold nanoparticles leached polymeric membrane in aqueous dispersion of *Candida bombicola* cells in water for 4 h. It was seen that hardly any number of the cells attached to the polymeric surface. This confirmed the role of hydrophobic gold nanoparticles embedded in the polymeric membrane were responsible for the attachment of the cells to the surface of the nanogold membrane.

Figure 4.B.7. (A) and (B) The SEM images after the immobilization of the *Candida bombicola* cells on gold nanoparticles leached polymeric membrane.
4.3.6. Synthesis of sophorolipids

Enzymes of the cytochrome P450 monooxygenase family are known to metabolize hydroxylation of arachidonic acid. Expressions of cytochrome P450 have been documented in liver, kidney and cerebral microvasculars. The earlier biochemical studies of the cytochrome P450 dependent arachidonic acid monooxygenase reaction showed that catalytic activity turnover was NADPH dependent. This was an advantage of using whole cells, which limited us to use cofactors such as NADPH for the synthesis of sophorolipids. Moreover, the advantage in the use of immobilized whole cell system with cell bound activity was that this obviated the need for enzyme extraction and removal of the unwanted macromolecules released during the extraction process which were laborious and expensive.

Candida bombicola yeast cells are known to produce extracellular biosurfactants known as sophorolipids. Sophorolipids are produced as a mixture of acidic and lactonic forms. Sophorolipids obtained from the Candida bombicola yeast cells; the lactonic form represented the largest fraction of the products. Scheme 4.B.1. has shown the transformation of arachidonic acid using Candida bombicola yeast cells to the acidic and lactonic form of the sophorolipids (Scheme 4.B.1. step 1). Thereafter, acid hydrolysis of these sophorolipids yields 20-HETE (Scheme 4.B.1. step 2). Nanogold membranes of the immobilized Candida bombicola yeast cells were reacted with arachidonic acid and the sophorolipids were isolated from the reaction medium and subjected to acid hydrolysis as described in details in the experimental section. The amount of cells immobilized on the 20 mg of hydrophobic nanogold membrane was estimated from the initial (~ $10^8$ cells/mL) and the final cell counts after immobilization as described in the experimental
section, and was estimated to be 0.3 mg. The immobilized yeast cells transformed 75 w/v % of the arachidonic acid to sophorolipid compared with the same amount of free cells in solution. The sophorolipids were separated by thin-layer chromatography (TLC) on standard Kiesel-gel 60 plates and were detected by mass spectroscopy. Figure 4.B.8. (A) and (B) have shown the mass spectra of the sophorolipids formed from arachidonic acid. Significant ions occurred at $m/z$ [710] and $m/z$ [728], and the structures were determined to be lactonic and the acidic forms of the diacetate respectively.

Figure 4.B.8. (A) and (B) Mass spectrum of the sophorolipids produced from the arachidonic acid. The sophorolipids are Lactonic (A) and Acidic (B) forms of diacetate as detected by mass spectroscopy.
The binding of the cells to the nanogold membranes was strong enough with marginal leaching of cells during the sophorolipid production. Hence, the immobilized cells on the hydrophobic nanogold membrane were reused for the five successive reaction cycles. The yeast cells bound to the hydrophobic nanogold membrane transformed 70 w/v % of the arachidonic acid to sophorolipid for the third cycle of reaction, while transformed 60 w/v % for the fifth cycle of reaction.

Figure 4.B.9. (A) and (B) have shown the low and high magnification of SEM images of different regions of Candida bombicola cells immobilized on hydrophobic nanogold membrane after one cycle of reaction (incubating in the reaction medium for 30 °C for 96 h). SEM images have shown the registry of the cells attached to the surface of the hydrophobic nanogold membranes. SEM confirms the fidelity of the yeast cells on the surface of the hydrophobic nanogold membrane after one cycle of reaction.

Figure 4.B.9. (A) and (B) Low and High magnification of SEM images of the immobilized Candida bombicola cells on the hydrophobic nanogold membrane after one reaction cycle

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Acid hydrolysis of the sophorolipid yielded 19-HETE and 20-HETE. The 20-HETE formed was analysed by gas chromatography. As the hydroxylated fatty acids released from the sophorolipids. **Figure 4.B.10.** has shown the partial mass spectrum of 20-HETE as detected by GC-MS. Significant ions occurred at \( m/z \) [406 \( M^+ \)], 391 [\( M^+ - 15 \)], 316 [\( M^+ - 90 \)], 304 [(\( M^+ +1 \) – 103)\(^17\), 23]. Selective ion monitoring also showed a prominent signal at \( m/z \) 103 [(\( CH_3 \)_3-Si-O^+\)-CH\(_2\)].\(^17\), 23 The mass spectrum thus clearly indicated that the hydroxyl group was at the C20 position and that the compound was 20-hydroxy 5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE). Confirmation of the identity of 20-HETE was obtained by the observed co-elution of 20-HETE standard by GC-MS with the isolated material.

The mass spectrum indicated a hydroxyl group at position C19. The mass spectrum of ions occurred at \( m/z \) 117 due to cleavage from C19 to C20; [(\( CH_3 \)_3-Si- O^+\)-CH-CH\(_3\)] and 73 [base ion; (\( CH_3 \)_3-Si] and loss of 220 [\( M^+ - 186 \)] due to rearrangement and loss of –CH=CH (\( CH_2 \)_3-CH[O^+\)-Si-(\( CH_3 \)_3]-CH\(_3\), 201 [\( M^+ - [131 + 74 (silyl group + H)] \)]. **Figure 4.B.11.** has shown the structures of the significant ions occurred of methyl ester silyl ether of 19-HETE. The compound was identified as 19-hydroxyeicosatetraenoic acid.\(^17\)
Figure 4.B.10. Partial electron impact mass spectrum of 20-HETE as detected by GC-MS. The compounds were extracted by from sophorolipids, synthesized by Candida bombicola cells and was identified as the methyl ester silyl ether of 20-hydroxy-eicosatetraenoic acid (20-HETE). The inset shows the structure of the methyl ester silyl ether of 20-hydroxy ecosatetraenoic acid (20-HETE)
Figure 4.B.11. Partial electron impact mass spectrum of 19-HETE as detected by GC-MS. The compounds were extracted by from sophorolipids, synthesized by *Candida bombicola* cells and was identified as the methyl ester silyl ether of 19-hydroxy-eicosatetraenoic acid (19-HETE). The inset shows the structure of the methyl ester silyl ether of 19-hydroxy ecosatetraenoic acid (19-HETE)
4.4. Conclusion

In this study, we have demonstrated the synthesis of hydrophobic nanogold membrane and thereafter used for the immobilization of *Candida bombicola* yeast cells driven by hydrophobic interactions between the cell walls and the ODA molecules. Enzymes of the cytochrome P450 monooxygenase family present in the yeast cells were used for the hydroxylation of arachidonic acid for the production of sophorolipids. The binding of the cells to the nanogold membrane was strong enough to prevent the leaching of the whole cells from the surface. Hence the whole cells immobilized membranes were reused for the biotransformation.
4.3. References


