Micro Emulsion Based Gel as a Host for Enzymatic Separation and Catalysis

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Certificate of Examination

This is to certify that the dissertation titled "Micro Emulsion Based Gel As a Host for Enzymatic Separation and Catalysis" submitted by Himanshu (Reg.No. MS15082) for the partial fulfilment of BS-MS dual degree programme of the institute, has been examined by the thesis committee duly appointed by the institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Subhabrata Maiti Dr. Santanu Kumar Pal Dr. Raj Kumar Roy

(Supervisor)

Dated: May 4, 2020

Declaration

The work presented in this dissertation has been carried out by me with Dr. Subhabrata Maiti at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Himanshu

(Candidate)

Dated: May 4, 2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Subhabrata Maiti

(Supervisor)

Acknowledgement

This research work would not have been completed without the encouragement and help of several people. I would like to thank each of them for their help, inspiration and cooperation.

I am very much thankful to Dr. Subhabrata Maiti for his valuable guidance and encouragement.

I would also like to thank Akshi Deshwal for providing indispensable advice, information and support on different aspects of my work.

Also, I thank my family and friends for their unwavering support.

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List of Abbreviations

CTAB	Cetyltrimethyl ammonium bromide
RM	Reverse Micelle
M.E.	Micro-emulsion
MBG	Micro-emulsion Based Gel
HRP	Horseradish Peroxidase
AG	alpha glycosidase
Gox	Glucose oxidase
H2O2	Hydrogen peroxide
CV	Crystal Violet
ODA	o-dianisidine
TMB	Tetramethylbenzidine

Abstract

The present study contains something phenomenal about Micro-emulsion based gels. This work involves formation of MBG (Micro emulsion Based Gel) via addition of water or aqueous sugar solutions in quiet high amount. The formation of thermally stiffening CTAB-micro-emulsion based gel (MBG), showing nano-confinement effect of carbohydrates in terms of micro-viscosity and hydrodynamic diameter of the reverse micelle. Upon heating, the mechanical strength of the gel increased up to 5 times. Also, advantage of this gel as efficient columnar bioreactor for entrapped enzymes (HRP) towards both hydrophilic and hydrophobic substrate in multiple runs has been elaborated. Finally, the superior catalytic ability of this MBG towards thermophilic α-glycosidase in multiple cycles at 60 °C has been demonstrated.

Quiet interesting properties revealed during MBG formation with the help of sugars like glucose, sucrose and fructose. Here these sugars might be acting as gelling agents making a quiet H-bond network resulting into gel. MBG also get influence by surrounding temperature, which upon heating give astonishing results in the field of rheology. It also can be used as a biocatalyst for the synthesis of 2 substituted Benzoxazoles via Oxidative Cyclization.

Chapter 1

Introduction

1.1Reverse micelles

Reverse micelles are a system of bulk organic solvent where an amount of water and amphiphile molecules (surfactant) are dissolved to form a single optically isotropic and thermodynamically stable liquid solution. The surfactant molecules form a monomolecular layer around the nanometer-sized water droplet with hydrocarbon tails facing the organic solvent and polar head-groups pointing inwards (Fig. 1).

According to Danielson and Lindman [1], the thermodynamically stable isotropic liquids obtained from this mixture usually are termed as **"Micro emulsion"** and consist of one or more phases which coexist separately, but are in balance with each other [2], e.g. water continuous (oil-in-water), oil continuous (water-in oil), bi-continuous (middle phase) [3]. The scientific recognition of micro-emulsion dates from 1943 (Hoar and Schulman)[4], but the term was first used by the latter author only in 1959 to describe the transparent solution of the multiphase system formed by water/oil/surfactant (w/o/s) mixture [5].

Most of the structural features of RMs are inducted and controlled by the ratio between the polar phase-to-surfactant concentrations. As water is generally the polar phase used to obtain RMs, this parameter is termed as degree of hydration and is considered even more relevant to describe the system features than the water content [6]. It is universally defined as the molar ratio of water-to-surfactant:

$W_0 = [H_2O] / [\text{Surfactant}]$

Water-in-oil reverse micelle

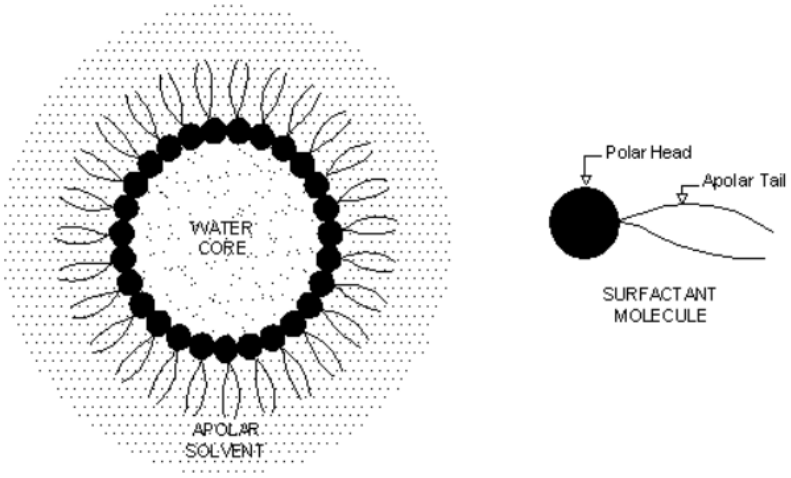


Figure 1 Showing molecular arrangement surfactant molecules in case of water in oil reverse micelle.[40]

Oil in water Micro-Emulsion

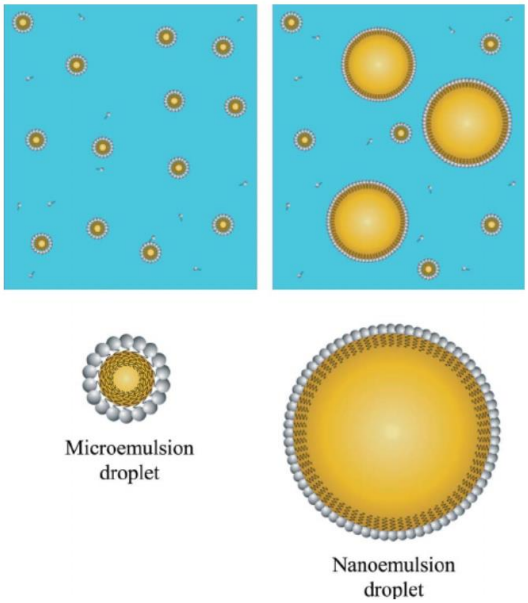


Figure 2 Showing the molecular arrangement surfactant molecules in case of oil in water. [41]

1.2. Application of Micro emulsions

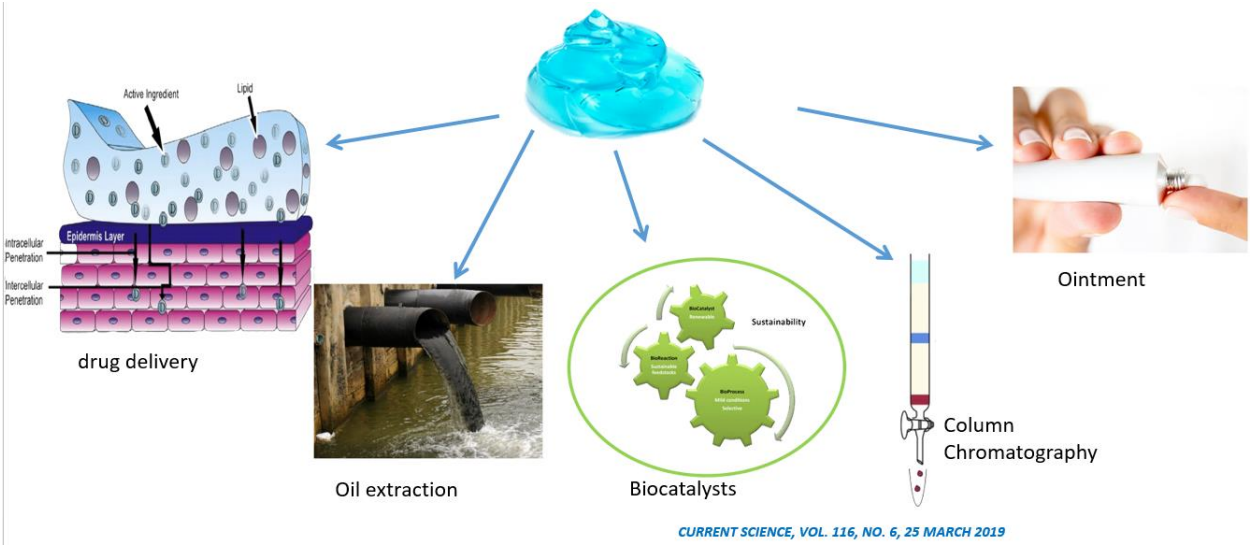


Figure 3 Showing the various application of micro-emulsion based gel

1.2.1 *Micro emulsions as cleaning systems*

One of the application of micro emulsions is their use as cleaning systems. The industrial cleaning processes involve a simultaneous removal of hydrophobic soils, such as grease, oil, etc. and hydrophilic contaminants like salt, pigment, protein, etc.

Conventional solvent-based systems for hydrophobic soil removal have been chlorinated hydrocarbons. Basically the uniqueness of micro-emulsion systems in detergency should be in their low interfacial tension towards external phases either aqueous or oil phases. The great advantage of applying a micro emulsion in the process would be to facilitate the solublization of both hydrophobic and hydrophilic components simultaneously. In this way a two-step process could be done in one step.

In industrial cleaning processes different kinds of organic solvents are in daily use .However a final breakthrough of M.E. in cleaning fields depends on many parameters such as price, chemical performance and compatibility with existing machines. The use of micro emulsions in wash/cleaning processes has been summarized by several authors (see Refs. [7, 9-17]).

1.2.2. Enhanced oil recovery (EOR)

The most promising application of micro emulsions is in oil recovery from oil reservoirs, it just because they has a surfactant showing detergency properties .Having low interfacial tension that also make them useful for this purpose.

This oil recovery process is quiet useful in oil exploration process in petroleum industries .There are many other artificial means to obtain oil from reservoirs but the use of ME based systems is gaining considerable amount of interest in these days.
Surfactant based systems like MEs have potential in oil recovery process which are more economically viable than other chemical systems. [Nazar et al., 2009]

1.2.3. Micro emulsions as Media for Chemical reactions

Catalysis in micro emulsions represents an alternative to phase transfer catalysis [18] and other techniques [19] for reaction of water insoluble organic substrates with water soluble reagents. A micro emulsion can physically catalyze or inhibit chemical reactions by interfacial or biphasic solubilization, and confine mentation.
Because of biphasic nature of micro emulsion systems, they are able to solubilize a no. of compound irrespective of their nature. [20]. Many reactions can be done in these systems like ester hydrolysis, photochemical reactions and other enzyme catalyzed reactions [8, 23,24],. And also they can be useful for chemical synthesis purpose. [30, 32-35].

1.2.4. Using MBG for the Synthesis of 2-Substituded Benzoxazoles

2-Substituted benzoxazoles are important scaffolds due to their occurrence within a wide range of biologically active natural products and pharmaceutical agents. And significant effort have been focused on the development of new synthetic procedures for generation of these heterocyclic compounds. Benzoxazoles are synthesized by the oxidative cyclization of phenolic imines mediated by strong oxidants working in concert with transition metal catalysts such as Pd, Cu, Fe, Ni, Ag, and Ru.

Due to which it have some toxic metal catalysts and production make it expensive. Therefore a milder and more efficient method to prepare these compounds would be highly desirable.
Bio-catalysis is an important tool sued in environmentally friendly and sustainable processes for the synthesis of organic molecules. Mining for new types of organic reactions that can be catalysed by proteins has drawn much attention in recent years and has dramatically expanded the application of proteins within the field of organic chemistry.

In the reference they have done their studies with different Biocatalysts, one of them was using Hemoglobin, in which they have reported an efficient and mild 2-substituded benzoxazoles. With that keeping in mind I had a thought of using **MBG** as a bio-catalyst for the same reaction for 2-substituted benzoxazoles.

1.2.5. Micro emulsion Based Gel (MBG)

The MBG system itself a micro emulsion along with presence of gelling agent like gelatin. Issue, micro emulsion based gel (MBG) (first demonstrated by Luis et al. in 1986). Although it has some advantages over micro emulsions like [35]:

- These gels have vast network thereby having large loading capacity of drug.
- These have better stability in comparison to creams and other transdermal powders.
- There is no need of any specific instrument for their preparation i.e. preparatory feasible and has low production cost.
- The gelled micro emulsion provides better product separation and enhanced reusability in comparison to ME.
- There other applications are quite similar to micro emulsion like catalysis with and without enzymes.

1.2.6. Bio-catalysis in MBG

As micro emulsions are not that much efficient in biocatalysts as much MBG because of less effective product recovery and much lesser reusability with the same enzyme. Many micro emulsions can be gelled by adding gelling agent. Enzyme – containing MBGs having gelatin are rigid and quiet stable in non –polar organic solvents, thereby can be used for bio-transformations in organic media. [36-38].

Chapter 2

Results and Discussion

This chapter involves experimental techniques and procedures used to study the biocatalysts using micro emulsion based gel and can be used as a Bio-catalyst. In the following results I've reported how thermally stiffening CTAB-micro-emulsion based gel (MBG) is forming. Mechanical strength of the gel increased up to 5 times upon heating.

2.1 Experimental Methods:

2.1.1 UV-Vis Spectroscopy:

UV-Vis studies were performed using Varian Cary 60 (Agilent technologies) spectrophotometer. The proton transfer catalysis was carried out by following the product formation at 380 nm. Total reaction volume in the cuvette was fixed at 1 ml and cuvette of path length 1 cm was used for the entire kinetic study. All measurements have been performed at 25 ^{o}C.

2.1.2 Fluorescence Spectroscopy:

Fluorescence measurements were performed using SHIMADZU Model RF-6000 Spector-fluorophotometer.

2.1.3 Optical and Fluorescent microscopy:

The optical and fluorescence microscopic images were collected using Zeiss Axis Observer 7 microscope having Axio Cam 503 Mono 3 Mega pixel with ZEN 2 software.

2.1.4 Transmission Electron Microscopy:

The Transmission Electron Microscopy images were taken on JEOL JEM-F200 microscope. Staining of the vesicle was done using 1% phosphomolybdic acid solution.

2.1.5 Dynamic Light Scattering:

The Dynamic Light Scattering (DLS) data was recorded on Malvern Zetasizer Nano-ZS90.

2.1.6 Rheology data:

Rheology data was recorded on Anton-Paar MCR 302 Rheometer.

2.1.7 Synthetically Study of Benzoxazoles Preparation:

Preparation and purification of Phenolic imine (Schiff Base) with the help of column chromatography, and analyzed using 400 MHz NMR.

2.2 Experimental Procedure:

2.2.1. RM formation:

It has been synthesized as reported in literature ref [3]. CTAB (50 mM)/iso-octane/*n*-pentanol/water micro-emulsion system at the required *z* and W_0 values. The microstructural parameter z and W_0 have been defined as the molar ratio of [co-surfactant]/[surfactant] and [water]/[surfactant], respectively. For instance, CTAB (36.4 mg) was dispersed in isooctane in a 2 mL volumetric flask, to which the calculated amount of co-surfactant was added to attain the corresponding z (= [co-surfactant]/[surfactant]) value and shaken vigorously. Finally, water or carbohydrate solution was added (to reach the corresponding W_0), and the whole suspension was vortex to obtain a clear homogeneous solution of CTAB (50 mM)/isooctane/n-hexanol/water or aqueous carbohydrate solution reverse micelle. Different alcohols were used as co-surfactant like butanol, pentanol, heaxanol, heptanol and octanol. W_0 range for reverse micelle formation at fixed z = 10 with different co-surfactant have been illustrated in the table given below (Table 1+2).

2.2.2. W_0 range of the RM formation in absence and presence of carbohydrate solution:

Table 1. Values of W_0 range in clear reverse micelle[a] forming zone and the formation of opaque gel with different co-surfactant at fixed z = 10.

Alcohol	**W_0 range where reverse micelle forms**	**Opaque Gel formation range**
Butanol	16-60	Not formed
Pentanol	16-60	80 – 140
Hexanol	16-66	Not formed
Heptanol	16-60	Not formed
Octanol	Not formed (always cloudy)	-

[a] [CTAB] = 50 mM, [co-surfactant] = 500 mM. After mixing all of the constituent's vortexing was done for minimum 5 min to ensure visible clarity in the formed solution.

Table 2. Values of W_0 range in clear reverse micelle[a] forming zone and the formation of opaque gel in absence and presence of carbohydrate solution of different concentration (250, 500 and 1000 mM) with only n-Pentanol as co-surfactant at fixed $z = 10$.

Sugar	**Visibility (W_0)**	**Opaque Gel (W_0)**
Only Pentanol (no sugar)	16-60	80-140
Glucose solution [250 mM]	12-66	90-140
Fructose solution [250 mM]	12-60	90-140
Sucrose solution [250 mM]	12-84	100-144
Glucose solution [500 mM]	12-72	100-144
Fructose solution [500 mM]	12-72	100-140
Sucrose solution [500 mM]	12-84	104-144
Glucose solution [1000 mM]	12-102	120-160
Fructose solution [1000 mM]	12-90	120-150
Sucrose solution [1000 mM]	12-110	130-180 (Clear Gel)

[a] [CTAB] = 50 mM, [n-pentanol] = 500 mM. After mixing all of the constituents' vortexing was done for minimum 5 min to ensure visible clarity in the formed solution. For carbohydrate containing MBG, instead of water, aqueous carbohydrate solution of a particular concentration was added.

2.2.3. Optical images of vials:

These are the images of vials turning to MBG with addition of water (Figure 4) or sucrose (Figure 6) with different co-surfactant (Figure5).

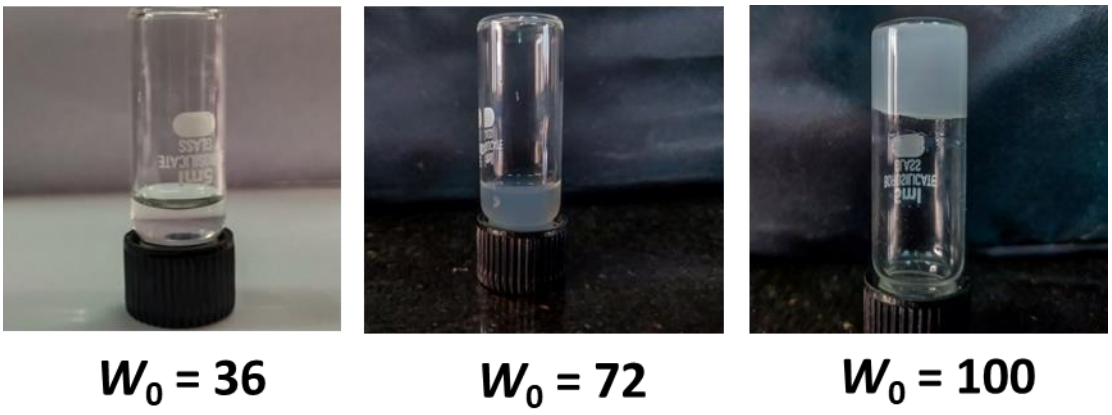


Figure 4. Optical images of inverted vials of CTAB (50 mM)/isooctane/n-pentanol/water reverse micelles at different W_0. 5 min vortexing was done after mixing of all the components. The images clearly demonstrate that at W_0 = 100, an opaque gel was formed when *n*-pentanol was used as co-surfactant.

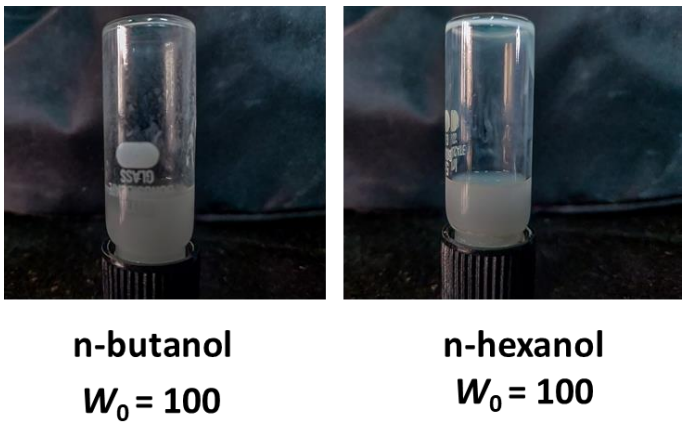


Figure 5. Pictures of inverted vials of CTAB (50 mM)/isooctane/n-butanol or n-hexanol/water reverse micelles at W_0 = 100 . 5 min vortexing was done after mixing of all the components. The images clearly demonstrate that at W_0 = 100, no stable gel was formed when *n*-butanol and n-hexanol were used as co-surfactant.

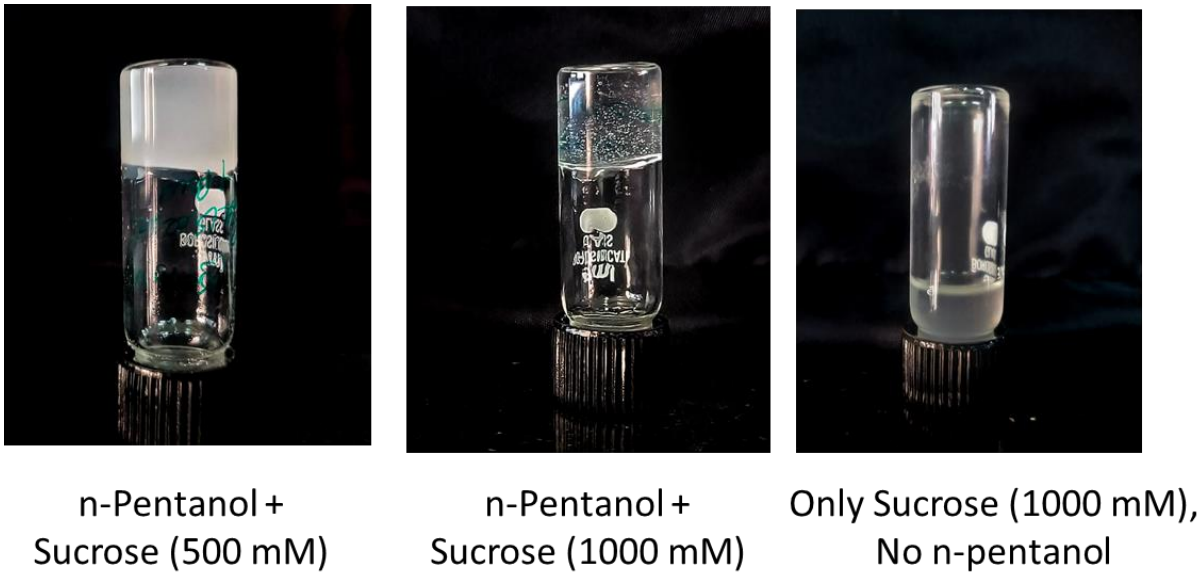


Figure 6. Images of inverted vials of CTAB (50 mM)/isooctane/n-Pentanol/sucrose solution reverse micelles at W_0 = 100 . 5 min vortexing was done after mixing of all the components. The images clearly demonstrate that at W_0 = 100, sucrose forms stable gel in presence of n-pentanol as co-surfactant.

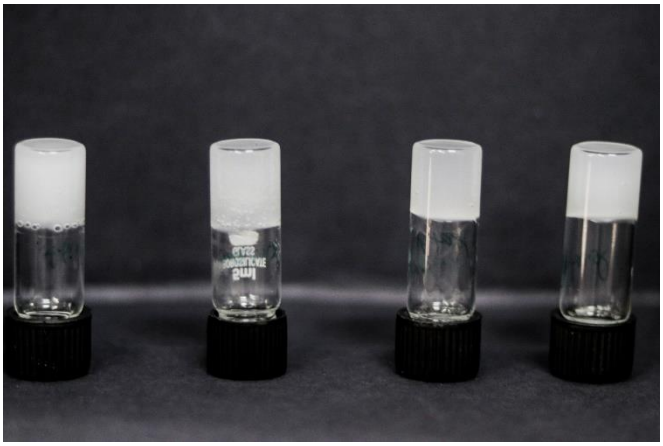


Figure 7. Naming from left:- MBG of water only (no added pentanol as co-surfactent),MBG of Sucrose 250mM, MBG Sucrose with added pentanol as cosurfactent, MBG of Pentanol only . Each is at W_0 =*100,* 5 min vortexing was done after mixing of all the components, stable gel was formed in each case.

2.2.3.1. Dynamic Light Scattering (DLS) Data

Keeping aim to observe the modulating effect of glucose and sucrose towards the size and viscosity of reverse micelle water pool having C_5-OH as the co-surfactant. Herein, we have conducted dynamic light scattering (DLS) experiment to observe the hydrodynamic diameter (D_h) of the reverse micelle in absence and presence of carbohydrate solution.

For the initial step we have measured only CTAB reverse micelle (in absence of any carbohydrate) solution at different W_0 at 24, 36 and 48) and found increasing D_h as 14.7 ± 1, 19 ± 2.1 and 27 ± 3.5 nm, respectively.

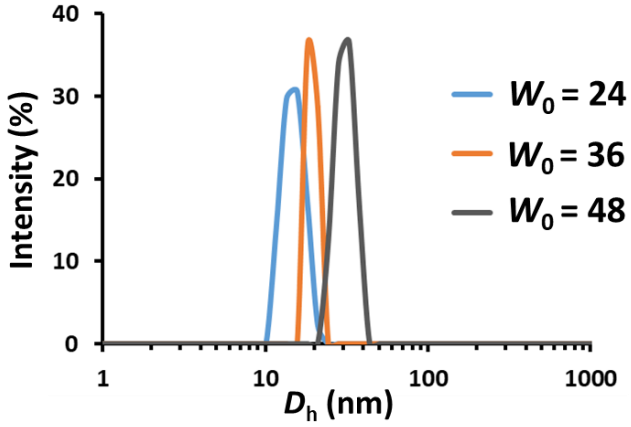


Figure 8. DLS plot showing the increase in D_h with increasing W_0 in CTAB (50 mM)/isooctane/n-pentanol/water solution.

But on addition of either glucose or sucrose resulted a considerable decrease in D_h value in each W_0 as observed in Figure 2a+.b For instance, at W_0 = 24, presence of 250, 500 and 1000 mM glucose solution in the water pool resulted a decrease in D_h from 14.7 ± 1 to 7.7 ± 1.1, 6.3 ± 1.4 and 5.9 ± 0.6 nm, respectively.

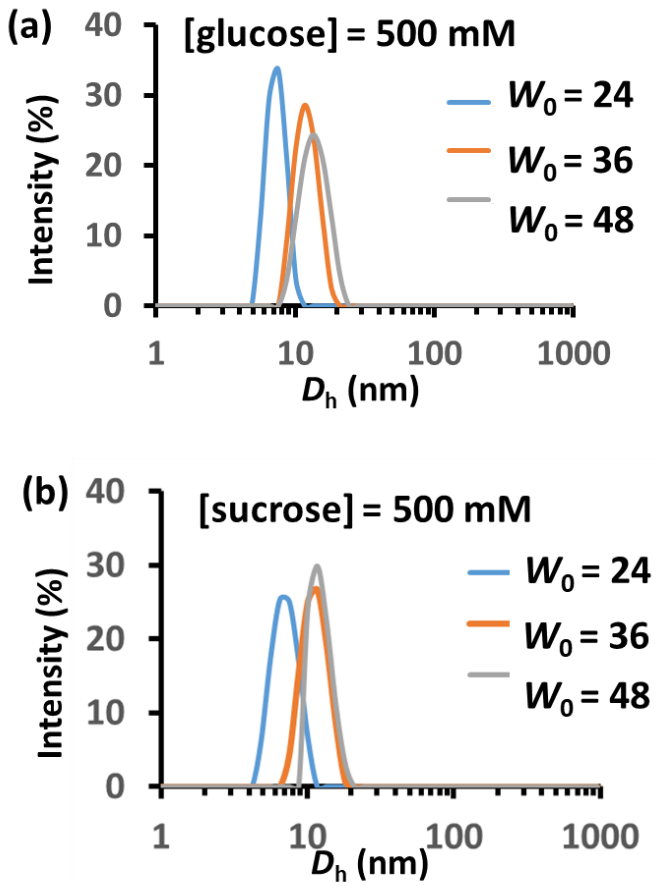


Figure 9. DLS plot showing the increase in D_h with increasing W_0 in (a) CTAB(50 mM)/isooctane/n-pentanol/500 mM glucose and (b) CTAB(50 mM)/isooctane/n-pentanol/500 mM 500 mM sucrose solution.

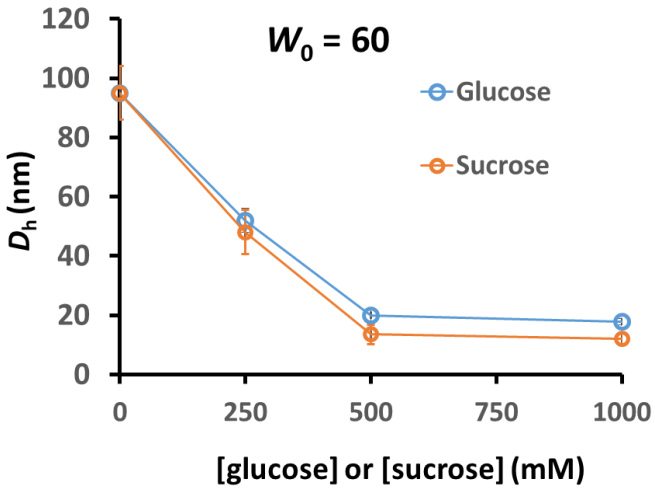


Figure 10. Hydrodynamic diameter (D_h) of reverse micelle at W_0 = 60, in absence and presence of glucose and sucrose solution of three different concentration (250, 500 and 1000 mM).

2.2.4. Fluorescence Studies

To observe the effect of glucose and sucrose in modulating the micro-viscosity of the water pool. For this purpose, we have used commonly used cationic fluorescence viscosity probe crystal violet (CV), which show enhanced fluorescence in viscous solution due to restricted rotational motion (Figure11).

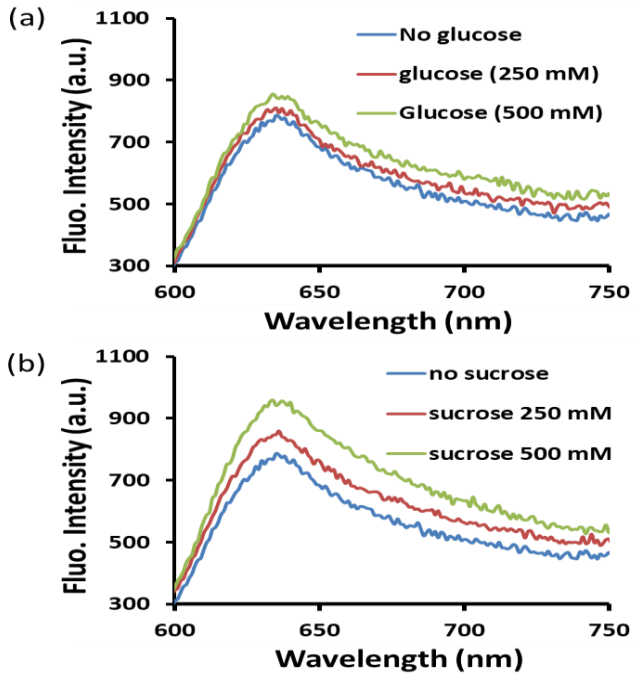


Figure 11. Representative fluorescence spectra of crystal violet (10 µM) in absence and presence of (a) glucose and (b) sucrose solution in water. Excitation wavelength = 575 nm, excitation and emission slit width = 10 and 5 nm. T = 25 °C.

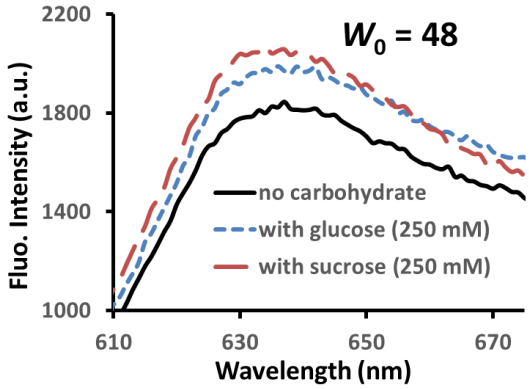


Figure 12. Fluorescence spectra of crystal violet (10 µM) in absence and presence of glucose and sucrose solution (250 mM) in CTAB(50 mM)/isooctane/n-pentanol/carbohydrate solution reverse micelle at W_0 = 48. Excitation wavelength = 575 nm, excitation and emission slit width = 10 and 5 nm. T = 25 °C.

2.2.5. Rheological Studies

To dipict the strenght of the MBG , I've done rheology , with in conconcentration of sucrose increases the mechanical strength of gel also increases, and there was a siginificance change in the data when the gel was heated at 60˚C . bellow are the figures that are explaing how data is diffrent in head and non heated MBGs.

2.2.5.1 Rheology study with only water based MBG having n-pentanol as the co-surfactant.

At 60 °C, G' value at linear viscoelastic (LVE) range of MBG has increased by almost 4-fold compared to 25 °C.

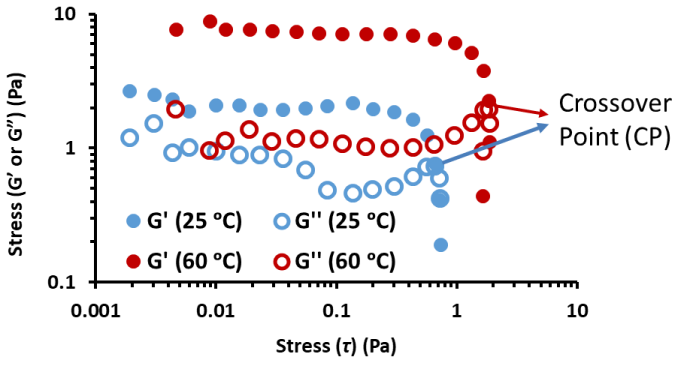


Figure 13. Plot of storage modulus (G') and loss modulus (G") as a function of oscillatory stress at constant angular frequency (ω = 0.1 rad/s) measured with CTAB based MBG (prepared by using of only water) with n-pentanol as co-surfactant formed at T = 25 °C and 60 °C. Experimental condition: [CTAB] = 50 mM, z = 10, W_0 = 120

2.2.5.2 Rheology study with sucrose solution (250 and 1000 mM) based MBG having n-pentanol as the co-surfactant .

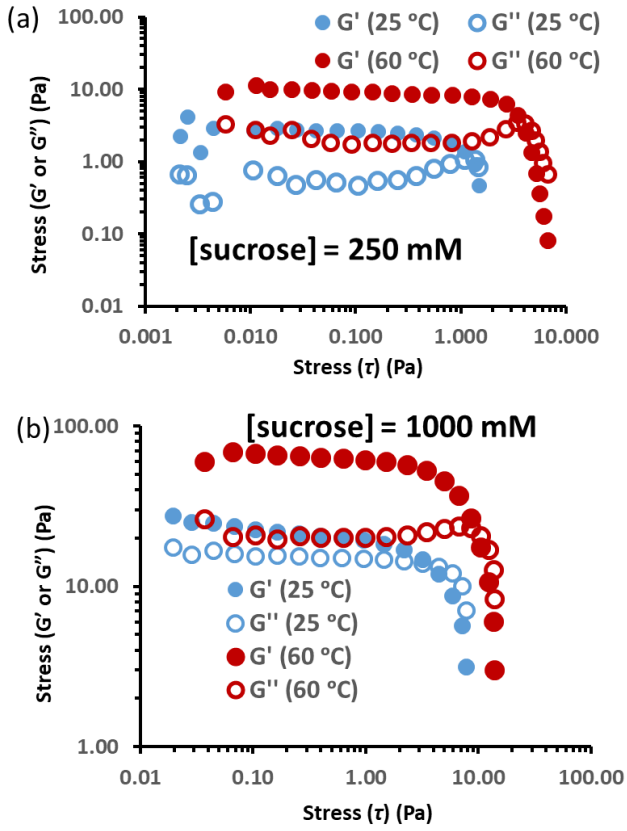


Figure 14. Plot of storage modulus (G') and loss modulus (G") as a function of oscillatory stress at constant angular frequency (ω = 0.1 rad/s) measured with CTAB based MBG prepared with (a) sucrose (250 mM) and (b) sucrose (1000 mM) solution having n-pentanol as co-surfactant formed at T = 25 °C and 60 °C. Experimental condition: [CTAB] = 50 mM, z =10, W_0 = 120.

2.2.5.3 Rheology study with glucose solution (500 and 1000 mM) based MBG having n-pentanol as the co-surfactant.

With increase in glucose concentration there is an increase in mechanical strenght of MBG but not as much as in case of sucrose. And also with the change of temprature there is slight increase in the effect but still not compareable to sucrose.

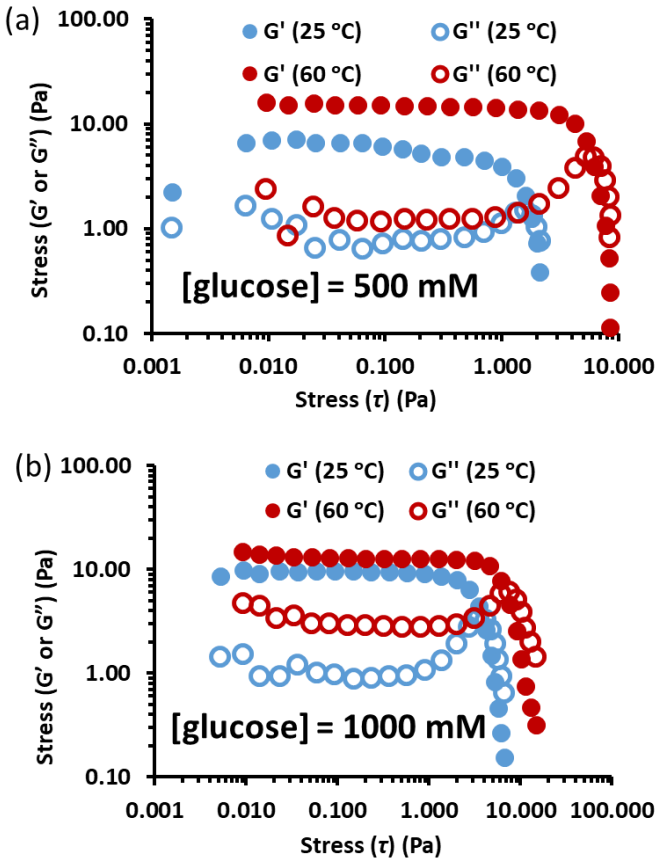


Figure 15. Plot of storage modulus (G') and loss modulus (G") as a function of oscillatory stress at constant angular frequency (ω = 0.1 rad/s) measured with CTAB based MBG prepared with (a) glucose (500 mM) and (b) glucose (1000 mM) solution having n-pentanol as co-surfactant formed at T = 25 °C and 60 °C. Experimental condition: [CTAB] = 50 mM, z = 10, W_0 = 120.

2.2.5.4. Rheology study with fructose solution (1000 mM) based MBG having n-pentanol as the co-surfactant.

With increase in the temprature, there is not much change in the mechanical strenght of MBG formerd from fructose addition.

And there is no prominent change in this effect on increasing the concentration.

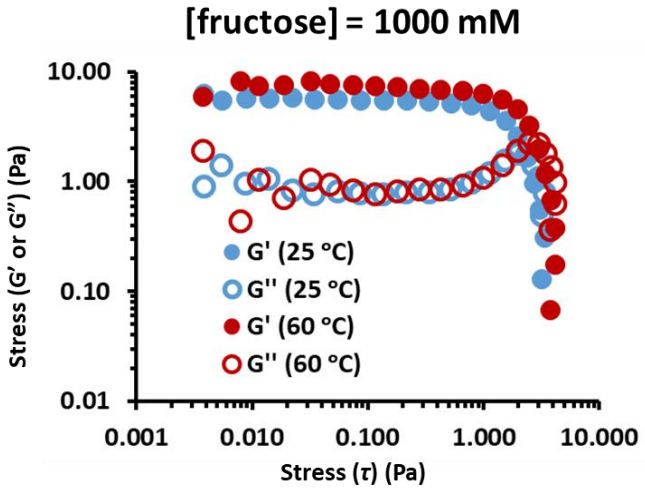


Figure 16. Plot of storage modulus (G') and loss modulus (G") as a function of oscillatory stress at constant angular frequency (ω = 0.1 rad/s) measured with CTAB based MBG prepared with fructose (1000 mM) solution having n-pentanol as co-surfactant formed at T = 25 °C and 60 °C. Experimental condition: [CTAB] = 50 mM, z = 10, W_0 = 120.

2.2.5.5. Summary of Rheology data

In a summarized way I can depict from the below figure 17 that mechanical strength of MBG is higher at elevated temperature that at Room temperature without any carbohydrates.

Also no other than “sucrose” show 3 fold increase in Mechanical strength when going from room temperature to 60˚C.

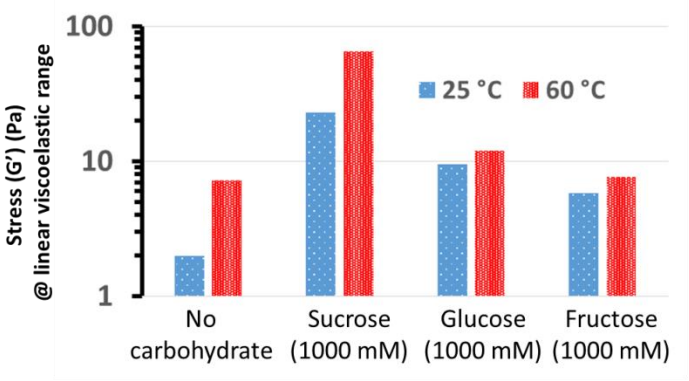


Figure 17. Storage modulus (G’) value at linear viscoelastic range at constant angular frequency (ω = 0.1 rad/s) measured with CTAB based MBG prepared with only water, glucose, sucrose and fructose (1000 mM) solution having n-pentanol as co-surfactant formed at T = 25 °C and 60 °C. Experimental condition: [CTAB] = 50 mM, z= 10, W_0 = 120.

2.2.6. Transmission Electron Microscopic (TEM) images:

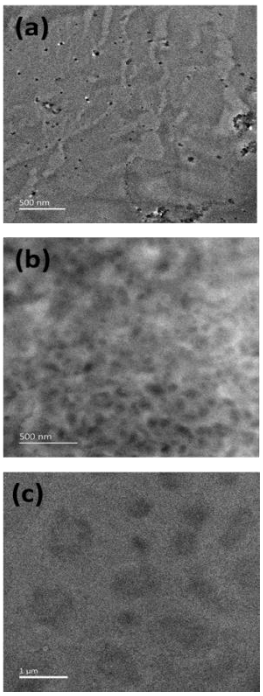


Figure 18. TEM images of MBG made of (a) water, (b) sucrose (250 mM) and (c) sucrose (500 mM) solution.

2.2.7. Optical Microscopic images:

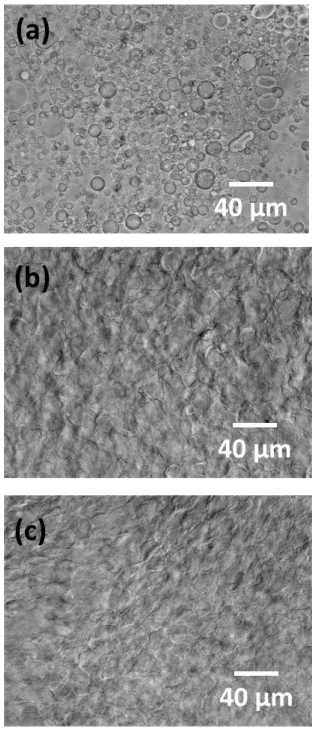


Figure 19. Optical microscopic images of micro-emulsion before forming gel made of (a) water, (b) sucrose (250 mM) and (c) sucrose (500 mM) solution. . Experimental condition: [CTAB] = 50 mM, isooctane as oil phase and pentanol as co-surfactant, z = 10, W_0 = 90.

2.2.8. Enzyme Separation study:

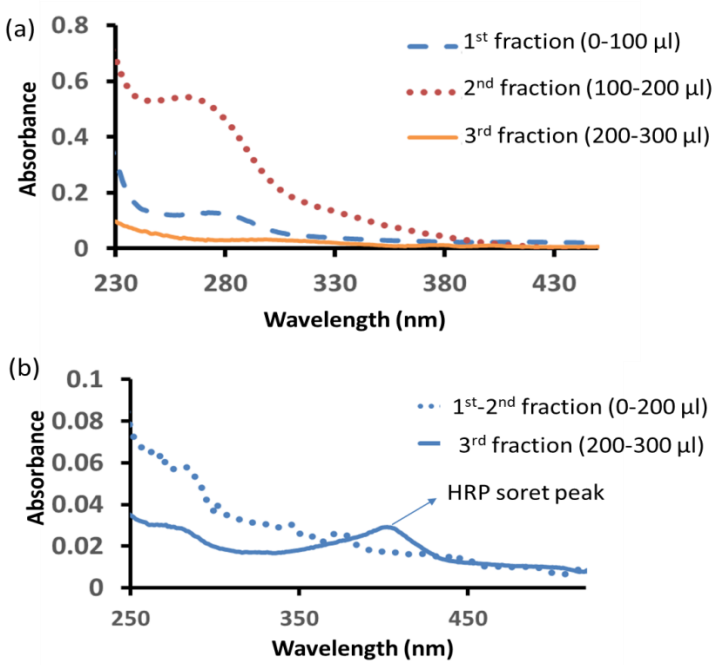


Figure 20. UV-spectra of different collected fractions of eluted (a) trypsin and (b) HRP through the MBG column when only trypsin or HRP was passed through it. 100 ul of water/ethanol (1:1) solution of trypsin or HRP having 60 µM concentration was passed through MBG of 5.5 ml in the column. In the MBG, oil phase is isooctane, [CTAB] = 50 mM, [sucrose] = 250 mM, z = 10 (n-pentanol as co-surfactant), W_0 = 120.

From the above figure 16, it is clear that trypsin being a water-pool solubilized enzyme passed through the column easily and almost all the trypsin gets eluted within 200 µl of eluted fraction. In contrary, HRP started to elute only in minor fraction after 200 µl of eluted fraction through the column. This clearly suggest that HRP being an interfacial solubilized enzyme has much higher propensity to get entrapped inside the column.

2.2.9. MBG column catalysis using HRP:

2.2.9.1 ODA as substrate:

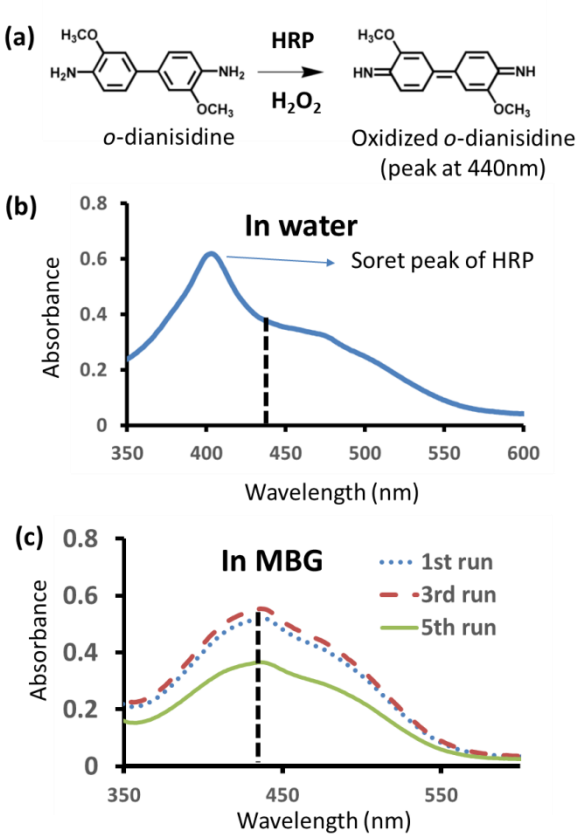


Figure 21. (a) Structure of the substrate o-dianisidine (ODA) and (HRP+H_2O_2)-oxidized product of ODA. (b) UV-vis spectra of the oxidized ODA when the reaction is carried in water in presence of HRP (1.2 µM), [H_2O_2] = 1 mM, [ODA] = 1 mM. (c) Representative UV spectra of eluted reaction product (oxidized ODA) from MBG, after 1st, 3rd and 5th run. 100 µl, HRP having 60 µM concentration was allowed to soak in the MBG of 5.5 ml in the column. Then 100 µl of water solution having ODA (1 mM) and [H_2O_2] = 1 mM, has been poured in the column having 5.5 ml of MBG and collected until the last drop (which takes around 5 min). This process has been done 5 times. In the MBG, oil phase is isooctane, [CTAB] = 50 mM, [sucrose] = 250 mM, z = 10 (n-pentanol as co-surfactant), W_0 = 120.

Calculation of the reaction product was calculated by following molar extinction co-efficient value of 11300 $M^{-1}cm^{-1}$, of oxidized ODA at 440 nm.[1] the eluted product amount was compared with only water and given in Figure 4c of the main manuscript.

Notably, in the eluted solution from the MBG, no peak of HRP (denoted by short band at 403 nm) has been detected. It clearly suggests that HRP has been retained inside the column.

2.2.9.2 TMB as substrate:

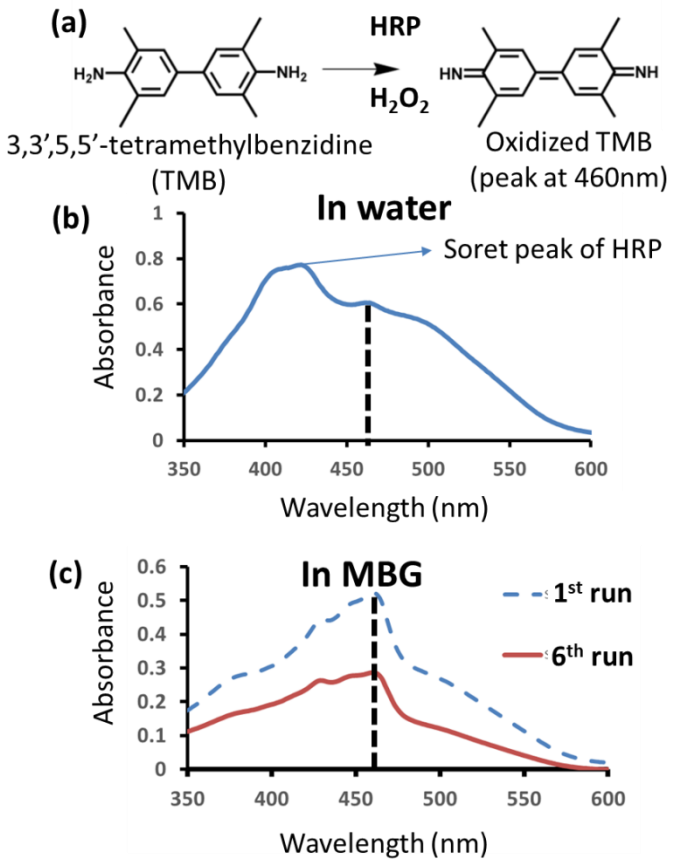


Figure 22. (a) Structure of the substrate TMB and (HRP+H_2O_2)-oxidized product of TMB. (b) UV-vis spectra of the oxidized TMB when the reaction is carried in water in presence of HRP (1.2 µM), [H_2O_2] = 1 mM, [TMB] = 1 mM. (c) Representative UV spectra of eluted reaction product (oxidized TMB) from MBG, after 1st and 6th run. 100 µl, HRP having 60 µM concentration was allowed to soak in the MBG of 5.5 ml in the column. Then 100 µl of ethanolic solution having TMB (1 mM) and [H_2O_2] = 1 mM, has been poured in the column having 5.5 ml of MBG and collected until the last drop (which takes around 5 min). This process has been done 5 times. In the MBG, oil phase is isooctane, [CTAB] = 50 mM, [sucrose] = 250 mM, z = 10 (n-pentanol as co-surfactant), W_0 = 120.

Calculation of the reaction product was calculated by following molar extinction co-efficient value of 59000 $M^{-1}cm^{-1}$, of oxidized ODA at 460 nm.[2] the eluted product amount was compared with only water and given in Figure 4c of the main manuscript.

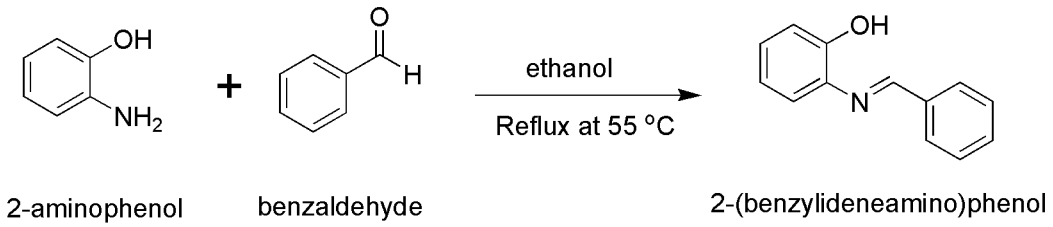
Notably, in the eluted solution from the MBG, no peak of HRP (denoted by short band at 403 nm) has been detected. It clearly suggests that HRP has been retained inside the column.

2.2.9. Preparation of Benzoxazoles:

2-Substituted benzoxazoles are very important scaffolds due to their occurrence within a wide range of biologically active natural products and pharmaceutical agents. And there are many significant effort have been made, which is focused on the development of new synthetic procedures for generation of these heterocyclic compounds.[42]

An efficient and environmental friendly protocol is what we seek for the synthesis of 2-substituted benzoxazoles, for that the primary reactant required was Schiff base, which is successfully synthesised. [42]

R1



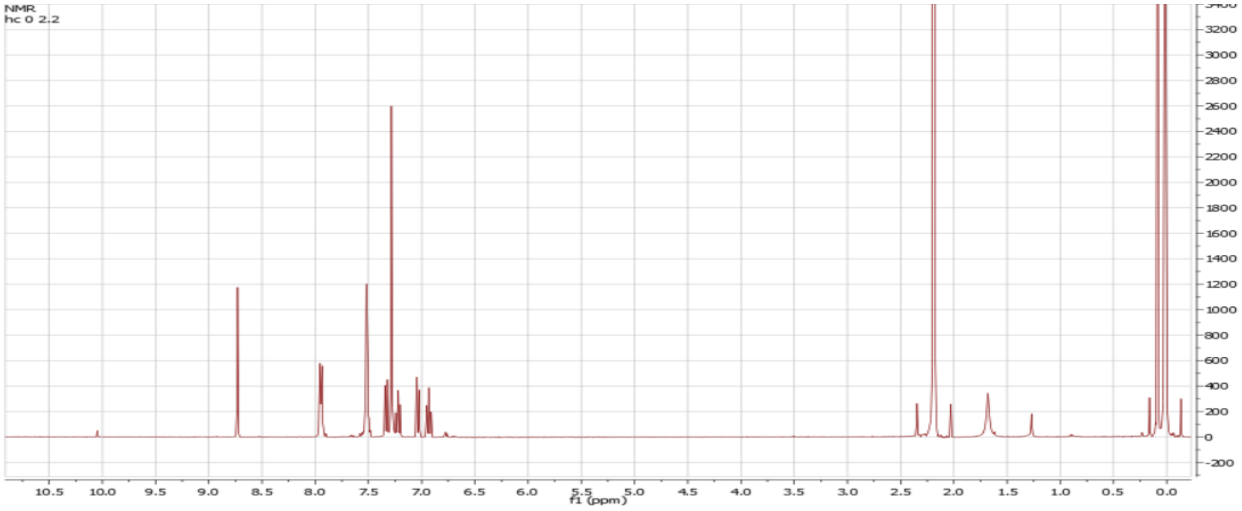
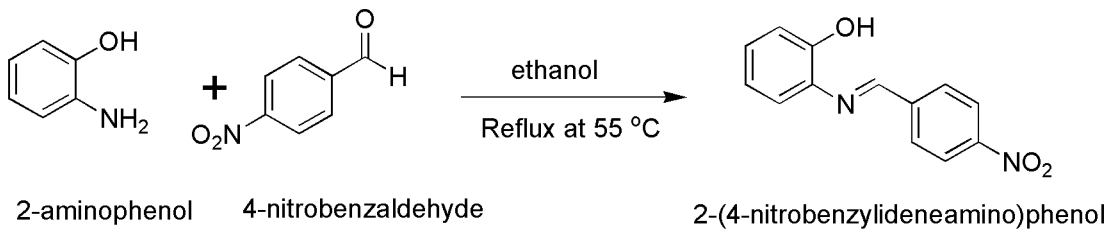


Figure 23. R1 is a reaction for phenolic imine, and above figure is NMR proof of a successful reaction

R2



2-aminophenol 4-nitrobenzaldehyde 2-(4-nitrobenzylideneamino)phenol

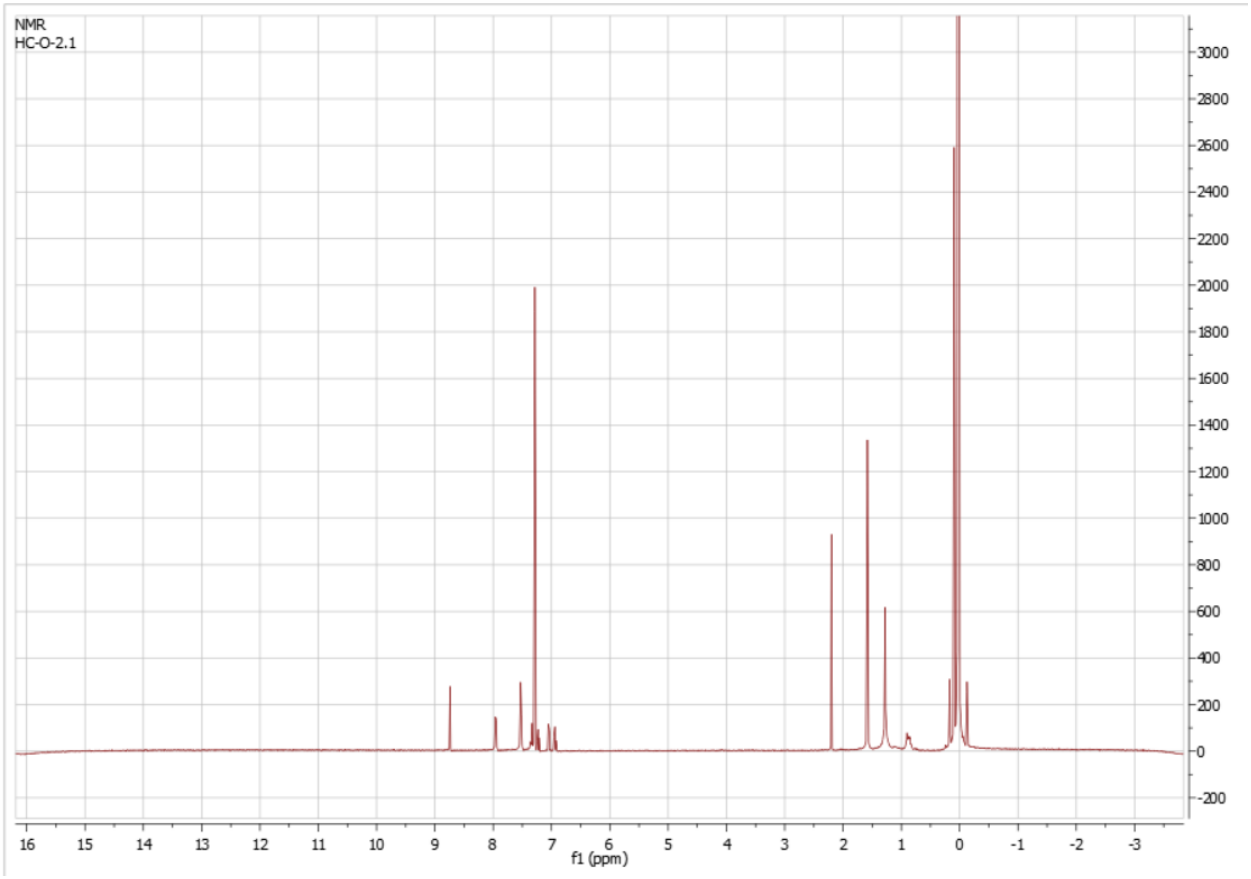


Figure 24. R2 is a reaction for phenolic imine with nitro-benzaldehyde, and above figure is NMR proof of a successful reaction.

In the reference publication they have reported the use of hemoglobin, which was used as a bio-catalysts for this reaction and they have obtained very high isolated yield (84-97%).

And with that I had a thought of using MBG as a Bio-catalysts, because it membrane bounded, and have high entrapment abilities.

R3

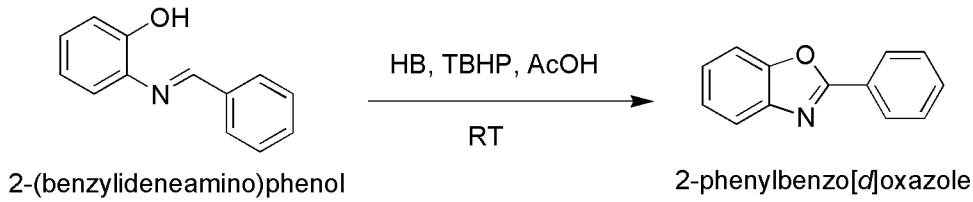


Figure 25. R3 is a reference reaction for the formation of 2-substituted Benzoxazoles using hemoglobin as a Bio-catalysts, failed to reproduce the results. HB refers to hemoglobin.

R4

Figure 26. R4 is a proposed reaction in mid-year presentation for the formation of 2-substituted Benzoxazoles using MBG as a Bio-catalysts. Stopped working on it shifted my direction to MBG's another application that is enzymatic catalysis.

Chapter 3

Conclusion

In summarized way the carbohydrates, specifically sucrose, can reduces the size of the water-pool diameter inside water-in-oil MBG and therefore enhances the viscosity of the confined environment. More importantly at high W_0 values of aqueous carbohydrate solutions MBG is able to entrap both hydrophilic and hydrophobic part.

Mechanical strength as depicted by rheology data, which shows it increases with increase in concentration of aqueous carbohydrate solution along with increase in temperature (60˚C), MBG become more pronounced in its effect of separation and catalysis. Furthermore, this MBG can act as a reusable host in the form of columnar bioreactor for enzymes like HRP with both water soluble and insoluble substrate. This work shows the carbohydrate confinement effect producing MBG which can further be utilized in studying some realm of systems chemistry.

Among all MBG the sucrose mediated one shows quiet phenomenal properties at room temperature and at elevated temperature at 60 degree C. Its strength was higher among all other MBGs comprised of glucose and fructose.

These strengthening properties can be justified with the all measurements like DLS, florescence and most importantly via Rheological measurements.

Underlining the reason sucrose led to increase in micro-viscosity of water-pool of RM, also with increase of sucrose concentration , interaction between polar head groups of CTAB and hydroxyl group of sucrose increase leading to formation of stable gel.

The florescence studies clearly shows more enhancement in micro viscosity in Micro emulsion in presence of sucrose rather than other sugars like glucose and fructose.

Among HRP and Trypsin only trypsin eluted out thereby confirming entrapment of HRP in MBG making it a good catalytic host.

Also there is 3-4 times enhancement of catalytic activity of HRP in MBG.

Thus it can be concluded from the following study that sucrose mediated MBG acted as a good catalytic host and has been proven quiet useful in separating membrane bound enzymes from non-membrane ones.

The mentioned properties regarding microscopic environment of MBG can be further exploited in systems chemistry arena.[39]

It can be used as a biocatalyst for 2-substituted Benzoxazole reaction.

Successfully reproduced Schiff base with different Benzaldehydes, however, work to carry out oxidative cyclization reaction for the formation of benzoxazoles using hemoglobin and MBG as bio-catalysts has not been completed.[42]

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