NOVEL METHODS TO PREPARE CROSS-LINKED ENZYME AGGREGATES (CLEA). CHALLENGING IMMOBILIZATION MODELS - UREASE AND PEPSIN

by

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To my beloved ma, grandma and grandpa

Always follow the white rabbit

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ABSTRACT

The common goal of various protein immobilization techniques has been to bypass the intrinsic drawbacks of utilizing free enzymes as catalytic materials in industry. Crosslinked enzyme aggregates (CLEAs), one of the most successful, easily and widely applicable techniques developed so far, has greatly improved the storage and operational stability of enzyme preparations as well as permitted their easy recovery and thus reuse. Involving the seemingly simple semi-specific chemical cross linking of protein aggregates forced out of solution, the general applicability of typical CLEA methods has occasionally been challenged by protein-specific anomalies, reflecting intrinsic structural and functional traits, altering the effectiveness of aggregation and crosslinkability, as well as the resultant bioactivity of the material.

In this work, the described limitations, have been addressed using two particularly CLEA-unfriendly protein starting materials, namely, native pepsin and urease.

In case of urease, conventional CLEA methods led to dramatically low aggregation and cross linking yields, and displayed statistically insignificant catalytic activity in the immobilized product. Critical breakthrough was achieved by enforcing protein aggregation via lyophilization

as opposed to routine precipitation. The subsequent crosslinking of the lyophilizate (yielding a CLEL) in a suitable antisolvent led to a much improved crosslinking yield and catalytic activity.

In case of pepsin, the problematic step was achieving covalent crosslinking by conventional CLEA methods, as pepsin bears a single surface lysyl residue and predictably was relatively unresponsive to all crosslinking attempts of surface amino groups. The problem was alleviated by appropriate choice of a rather large crosslinker, i.e., dextran polyaldehyde, and the use of the subzero crosslinking temperatures, therefore permitting the formation of the first ever catalytically competent pepsin CLEA.

Novel immobilized formulations presented herein, are expected to contribute as alternatives to many established industrially important applications, involving challenging protein systems. Furthermore, these also could be utilized to prompt greener processes, such as the syntheses of industrially important commodity compounds.

ÇAPRAZ BAĞLI ENZİM AGREGATLAR (CLEA) HAZIRLAMAK İÇİN YENİ METOTLAR. ZORLU İMMOBİLİZASYON MODELLERİ – ÜREAZ VE PEPSİN

Tuğçe AKKAŞ

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ÖZET

Çeşitli protein immobilizasyon tekniklerinin ortak amacı serbest enzimlerin endüstride katalitik malzemeler olarak kullanılmasındaki esas engelleri aşmaktır. Çapraz bağlı enzim agregatlar (CLEA), şimdiye kadar geliştirilmiş en başarılı ve uygulaması oldukça kolay tekniklerden biri olarak, enzim preparatlarının depolama ve operasyonel stabilitelerini iyileştirmekte olup, aynı zamanda geri kazanım ve yeniden kullanılabilmesine imkan vermiştir. Süreç çözünmüş proteinlerin agregat halinde elde edilip, yarı-spesifik olarak kimyasal çapraz bağlama adımlarından oluşmaktadır. CLEA metodunun genel uygunabilirliği bazı durumlarda protein türüne spesifik olan, yapısal ve fonksiyonel özelliklerine bağlı olarak oluşan anomalilerden dolayı sınırlı kalmaktadır. Bu durum agregasyon ve çapraz bağlama verimine, ayrıca sonuç olarak elde edilen biyoaktiviteye olumsuz yansımaktadır.

Bu çalışmada söz konusu olan sınırlamalar, özellikle CLEA süreci için uygunluğu fazlasıyla düşük olan pepsin ve üreaz proteinleri kullanılarak ele alınmıştır.

Üreaz durumunda, geleneksel CLEA yöntemleri önemli ölçüde düşük agregasyon ve çapraz bağlama verimlerine sebep olmuş, ve immobilize üründe ihmal edilebilir katalitik aktivite gözlemlenmiştir. Rutin çöktürme yerine liyofilizasyon yöntemi kullanılarak agregasyon

gerçekleştirilmesi bu soruna önemli çözüm getirmiştir. Liyofilizatların uygun antiçözücü içerisinde çapraz bağlanmasıyla yüksek çapraz bağlama ve sonuç katalitik aktivite verimlerine sahip çapraz bağlı protein liyofilizatlar (CLEL) elde edilmiştir.

Pepsin durumunda, tek serbest lizin grubu taşıyıp amino gruplarına yönelik çapraz bağlama denemelerinin zorlu olmasından kaynaklanarak, zorlu adım kovalent çapraz bağlama adımı olmuştur. Bu sorun, oldukça büyük bir çapraz bağlayıcı olan, dekstran polialdehit seçimiyle ve sıfır-altı çapraz bağlama sıcaklığı kullanılarak çözülmüştür. Böylece ilk katalitik olarak fonksiyonel olan pepsin CLEA üretimi gerçekleştirilmiştir.

Burada sergilenen yenilikçi immobilizasyon formülasyonları, özellikle zorlu protein sistemler durumunda, önemli endüstriyel uygulamalarda kullanılan geleneksel yöntemlere alternatif olarak katkı sağlaması beklenmektedir. Bunun dışında, bu çalışmada geliştirilmiş olan yöntemler, endüstriyel olarak önemli olan bileşik üretimi için yeşil sentez süreci oluşturulmasında kullanılabilir.

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Now it's the last page to write. This part is maybe more comforting but also more difficult than writing the thesis. I know this is going out of topic but I need to unburden my heart and write something about the most challenging but also most instructive and "Alice in Wonderland" style years of my life.

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As my angel mom just said: "Let your life always be in Wonderland. Who cares the others?"

Just believe in yourself and follow the white rabbit.

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LIST OF SYMBOLS AND ABBREVIATIONS

CLEA: Cross linked enzyme aggregates

CLEL: Cross linked enzyme lyophilizate

GC-MS: Gas chromatography – Mass spectroscopy

SEM: Scanning electron microscopy

DLS: Dynamic light scatter

DPA: Dextran polyaldehyde

TCA: Trichloro acetic acid

CHAPTER 1 Introduction

1.1 Protein Immobilization

While protein catalyst has been conventianally shown highly beneficial on a wide range of industrial, analytical and biomedical applications, the utilizability of native protein formulations is challenged by a number of factors including mechanical and chemical stability under conditions varying from those physiologically prescribed by the source of the particular protein and its specifications. The main aim of numerous protein immobilization techniques developed, has been to improve protein stability under conditions varying from the native proteins optimum but necessary for a given application, such as temperature, pH, ionic strength, organic solvent etc. Furthermore, immobilization should also achieve increased shelf life and provide reusability of the catalyst, while retaining catalytic activity [1-3].

Many approaches have been successfully attempted to achieve this goal over the years. The developed techniques can be generally classified as physical adsorption, encapsulation, and surface immobilization and cross linking [4, 5]. Out of these categories cross linking forms the method of interest in this work and will be discussed in further detail.

1.1.1 Crosslinking

Crosslinking is the process of chemically joining two or more molecules by a covalent bond. Covalent modification and crosslinking of proteins is achieved via various chemical reagents facilitating reaction with functional groups naturally occurring in proteins structure. These are protein amino acid side residues, namely amino-, carboxy- and sulfhydryl. The later is generally used in cases where specific modification is favored, while charged amino- and carboxy- groups due to their abundance on the surface of a globular protein are target to non-specific multiple covalent modifications. These alterations serve to stabilize the protein integrity by preventing disrupting conformational changes. Nevertheless, sub-optimal crosslinking type or degree may inhibit/decline native activity of the protein by directly altering of the interior residues responsible for binding or catalysis or by restricting necessary conformational mobility [6].

Primary amines are present at the N-terminus of a polypeptide chain (α-amine) and in the side chain of lysine (Lys) residues (ε-amine) and are conventionally subjected to modification with N-hydroxy succinimide esters, imidoesters and aldehydes. For the purposes of inter-protein conjugation, bifunctional crosslinking reagents are employed. In this study aldehyde type reagents have been employed.

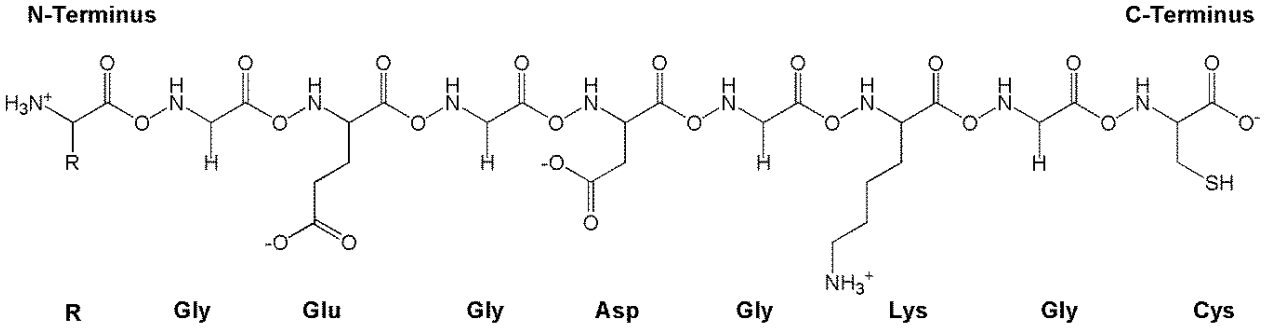


Figure 1-1 Common amino acid functional groups targeted for bioconjugation [7]

Glutaraldehyde is the most abundantly used reagent for the purpose [8]. In cases of proteins with less abundant surface lysine content, dextran polyaldehyde has shown higher yield. It also provides milder reaction conditions, and reduces toxicity risk, therefore preferred in many biomedical applications [9]. Yet another important rationale mentioned in literature is use of this crosslinker as an alternative to low molecular weight glutaraldehyde in order to prevent modification of lysine side residues, present in the active sites of many enzymes.

The reaction mechanism of aldehydes with amino residues is assumed to proceed through dehydration upon formation of Schiff bases intermediate (Figure 1-2). This assumption is the result of over simplification while in reality glutaraldehyde forms various species in an aqueous solution particularly depending on the pH value, therefore various reaction mechanisms are expected to contribute to the overall modification [10, 11]. Under general conditions the reaction is reversible and requires further reduction with sodium cyanoborohydride or sodium borohydride.

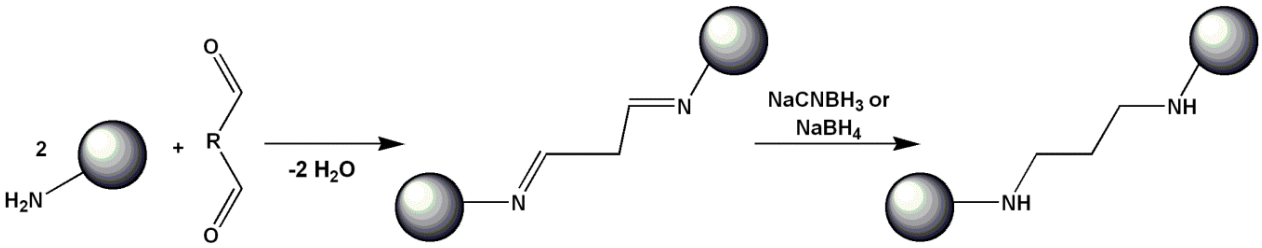


Figure 1-2 Reductive amination reaction of aldehydes

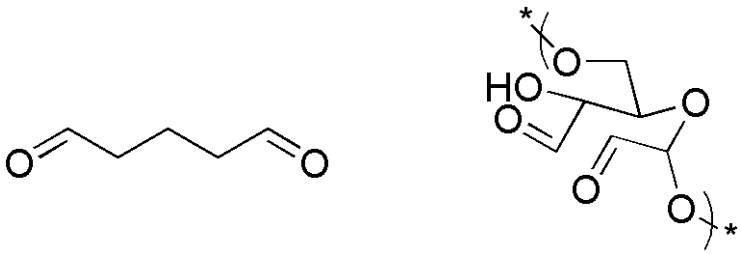


Figure 1-3 Structures of glutaraldehyde (left) and dextran polyaldehyde (right)

Carboxyl- residues are present at the C-terminus of a polypeptide chain and in the side chains of aspartic acid (Asp) and glutamic acid (Glu) and are reactive towards carboiimides, this technique has been widely applied in case of peptide synthesis.

Carbodiimides act through carboxyl group activation leading to zero length amide bond formation (Figure 1-4). Since o-acylisourea intermediate is unstable, the reaction is often aided by reagents such as hydroxysuccinimide that protect target carboxyl group through ester, which allows further conjugation with amino residue [6].

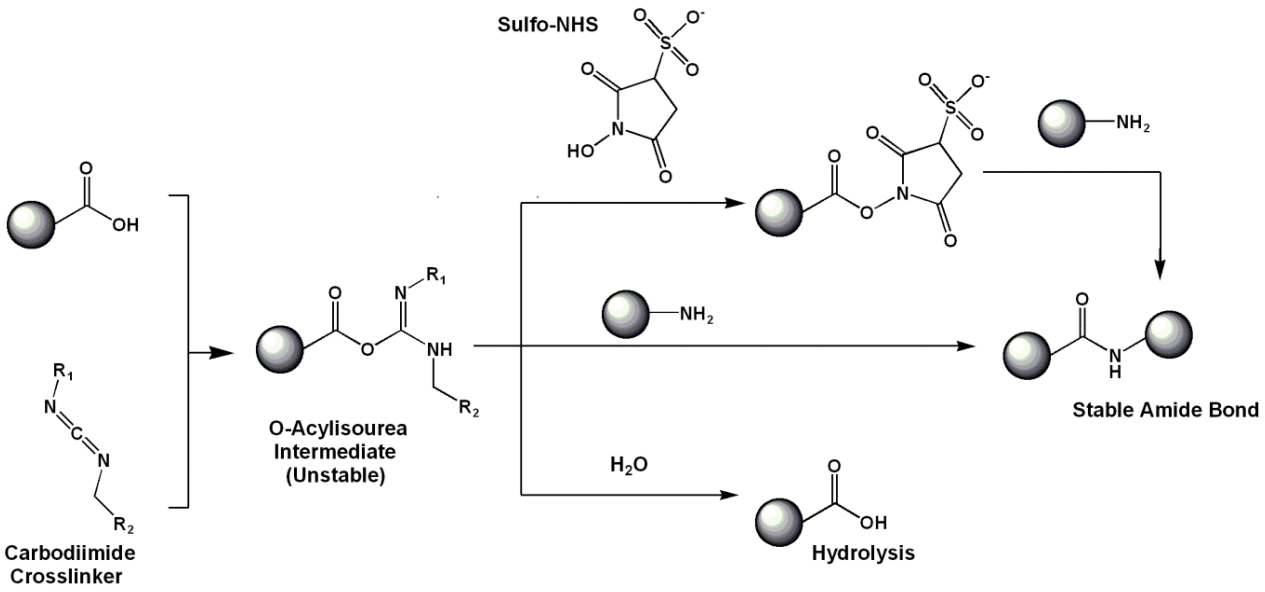


Figure 1-4 Carboxyl activation – amide formation

In case of heterogeneous reaction physical proximity of opposing groups is less probable, which makes the method less efficient, but with the use of the said aid or combined with amino- residue oriented crosslinker can prove very useful.

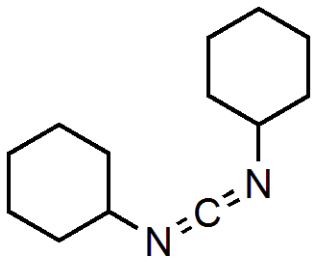


Figure 1-5 Structure of N,N'-Dicyclohexylcarbodiimide

While non-specific or semi-specific crosslinking of protein in solution state are effectively applied targeting many applications, the product often results in greatly diminished or inhibited catalytic activity. This can be readily explained by susceptibility of flexible proteins in aqueous

solution. This issue has been addressed by introduction of crosslinked protein crystals and aggregates in an exceptionally successful manner.

1.1.2 Crosslinked Enzyme Crystals (CLEC)

Crosslinked enzyme crystal formulations are one of the most efficient examples of mentioned crosslinking method, and have been developed since 1960s [12]. The technique was initially developed as the means of protein stabilization for X-ray diffraction studies. In the course of the study it has been realized that CLEC possessed retained and in many cases enhanced catalytic activity, nevertheless the follow up research has not been continued up to last two decades. Currently CLEC form the golden standard of crosslinked enzyme formulations [13, 14]. These provide an exceptionally stable formulation with advantage of very pure enzyme content, therefore providing high catalyst to weight ratio. That being said, formulations involve a very laborious synthesis process and require enzymes of very high purity, implying very high costs of large scale productions. Furthermore, the technique is obviously limited to only certain (crystallizable) enzymes.

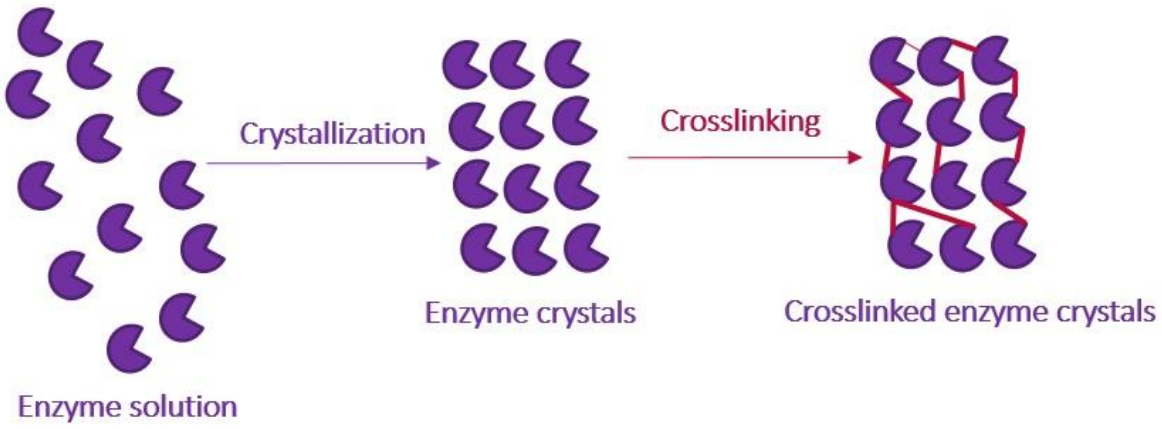


Figure 1-6 Illustration of CLEC formation

1.1.3 Crosslinked Enzyme Aggregates (CLEA)

Addressing the described drawbacks of CLECs cross linked enzyme aggregate technology has been pioneered by Roger Sheldon et. al. [15]. CLEA retain very good stability while based on a very general user friendly synthesis process which can also be applied to a very wide range of proteins. The process is also suitable for technical grade protein stocks, while in fact also facilitating further purification as a part of the process.

In a typical preparation, soluble monomeric protein starting materials is crash precipitated out of the solution, forming macroscopic aggregates. For this purpose saturated inorganic salt solutions are used, making use of salting-out principle. Just as well, water-miscible organic solvents (antisolvents) are employed. Other conventional protein precipitation techniques, such as polymer and isoelectric point precipitations have proved less efficient, but can be incorporated with the methods above during optimization.

The choice of precipitation medium is target protein dependent, affecting both aggregation yield and enzymatic activity of the end product. Efficiency of the further crosslinking procedure is also a factor. Co-precipitation and addition of protectants is employed to further stabilize the protein throughout crash precipitation step.

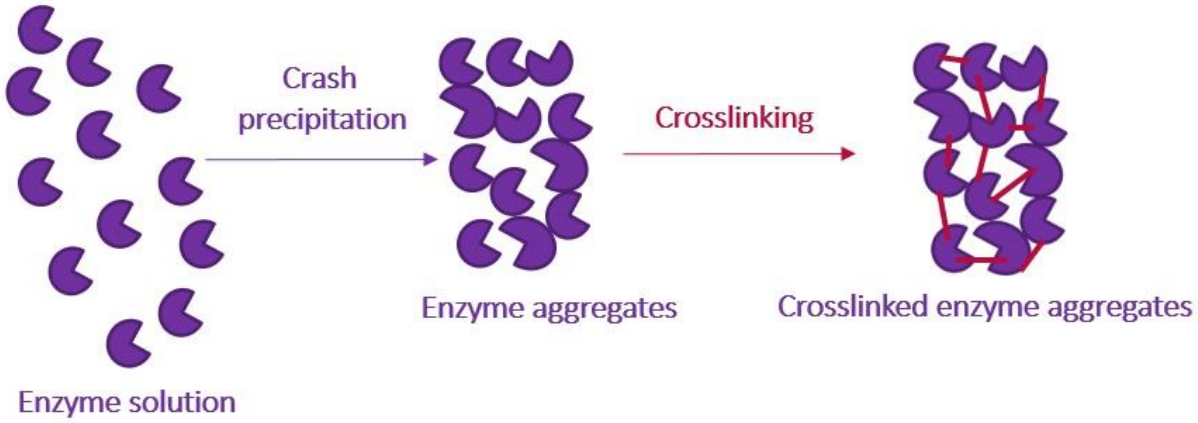


Figure 1-7 Presentation of CLEA production procedure

The obtained soft solids are generally further subjected to cross linking directly in the aggregation medium, using the suitable reagent, to yield final CLEA product. The aggregation and crosslinking steps are conducted in a manner that permites retention, and in many cases, improvement of biological activity.

1.2 Nanosizing and Alternative CLEA Production Methods

The topic of this study forms a part of TÜBİTAK 1001 project no 111M680 "Crosslinked Protein Nanoaggregates" [16]. Technique developed in-house, within the scope of this project, was inspired by the conventional CLEA methodology and aimed to address problems arising in micron and higher size heterogeneous catalyst systems, such as mass transport limitations, reduced access to catalytic centers, restricted catalytic turnover due to crosslinking. One approach to mitigate these issues has rested on limiting the particle size to the nanoscale. Various bottom-up approaches have been established, by bringing together individual protein units, yielding nanoscale enzyme particles. While effective, such attempts have generally proven very laborious, expensive, protein-specific, lossy, and impractical towards various target proteins [17, 18]. In contrast, herein this issue was successfully addressed with a generalized procedure suitable for wide range of proteins and applications, namely physical nanonization of crosslinked protein aggregate particles by application mechanical and hydrodynamic shear, thereof forming the first top-down approach in this area. The principle lies within limiting the particle size to the nanoscale so as to optimize substrate turnover, while retaining all the stability advantages associated with crosslinking.

In the course of this study conventional CLEA approach was pursued in synthesis of precursor materials. Optimization of these processes has been performed aiming to better accommodate following downsizing procedure.

Alternative formulations have also been developed, to address particularly challenging enzyme types in terms of aggregation and crosslinking capabilities aiming highly enhanced overall synthesis yield, and in some cases prevent dramatic loss of catalytic activity. Furthermore these formulations aided plausible alternative to conventional CLEAs, overall successful but yielding

suboptimal (less than 100%) production yields which could be observed on the examples of trypsin and chymotrypsin.

The case of particularly aggregation unfriendly proteins was partially resolved by solution-phase crosslink-assisted aggregation method. In which case the conventional procedure supplemented addition of very small amount of crosslinker to aqueous solution prior to precipitation and main cross linking steps [16].

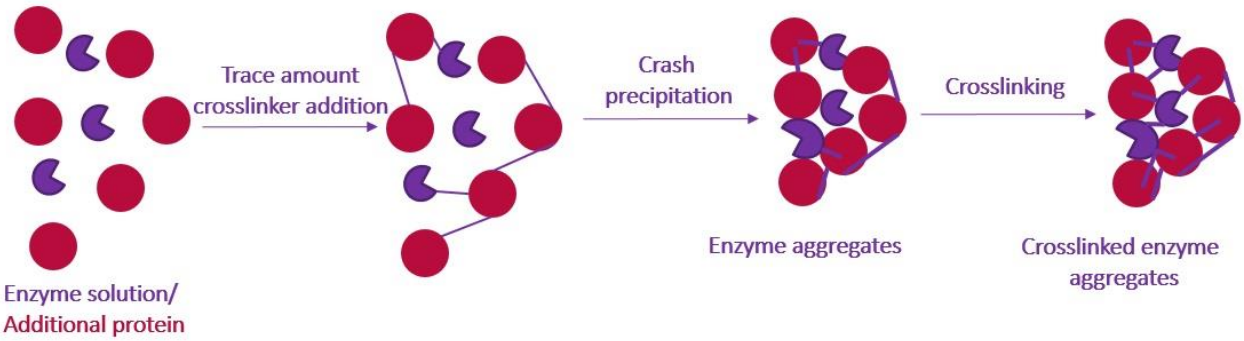


Figure 1-8 Presentation of solution-phase crosslink-assisted aggregation method

Use of lyophilizates in place of crash precipitated aggregates has been incorporated as the means of handling protein solutions that either showed low aggregation efficiencies and/or did not withstand aggregation step resulting in dramatic activity loss (Zakharyuta, A., PhD Thesis, Nanosized Crosslinked Protein Aggregates (nano-CLPA)). It was rationalized that the aggregate state could be achieved through lyophilization, as a conventional widely applicable technique, where crash precipitation did not lead to desirable result. In this procedure, optimally formulated protein solutions were lyophilized and immersed in a medium suitable for further crosslinking step, generally an organic solvent, yielding Crosslinked Enzyme Lyophilizates (CLEL). This new method, provided optimum process steps, has been noted efficient for all formulations tested, generally leading to higher overall yield, with more predictable enzymatic activity and easily handled final product for further manipulation.

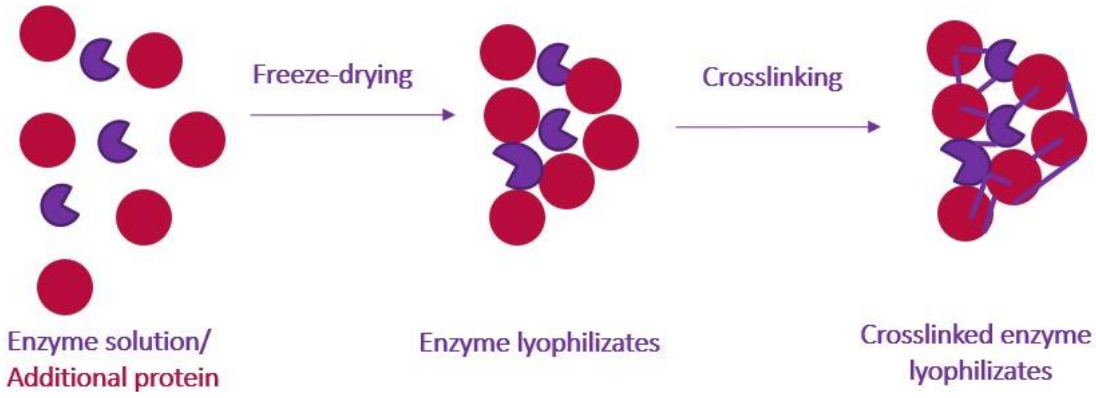


Figure 1-9 Presentation of CLEL formation procedure

These methods were generally conducted alongside co-precipitation incorporation; aid both aggregation and protection of protein structure to sustaining enzymatic activity, and optimization of crosslinker choice and physical conditions of the process.

It followed to reason that urease and pepsin formed ideal candidates for further optimization of CLEA/CLEL formulations, by incorporation of the described novel methods.

Both enzymes have no established covalent immobilization techniques so far, due to their structural anomalies:

The reason for the poor protein precipitability of urease was not clear, but the poor crosslinking outcome appeared to be related to an unusual structure, which discouraged surface functional group interactions with crosslinker [19].

Protein aggregation, in case of pepsin, proceeded routinely. The problematic step was achieving covalent crosslinking by conventional CLEA methods. The reason was again related to structure, as pepsin bears a single lysyl residue. Given that the formation of a crosslinked mass would demand two and at times three reactive groups per monomer, it was not surprising that pepsin was relatively unresponsive to all crosslinking attempts mediated by surface amino groups.

Development of optimum CLEA/CLEL formulations for pepsin and urease forms the focus of this work.

1.3 Applications of CLEA and nano CLEA

CLEAs form plausible alternatives as industrial biocatalyst systems, in terms of their economic and environmental benefits. The well explored application fields such as detergent, textile, leather industry, food, animal feed industry and biodiesel production and waste treatment are well suited for these formulations. More specific fields such as organic synthesis, sensory and diagnostic test enzymes, chromatography media, and artificial antibodies production are also benefiting from this method, with the largely growing need for stable biocatalyst throughout development of the related fields. Particularly the case of nano-CLEAs could potentially be in biomedical applications along with biosensors, including both systemic and local therapeutics, aiming topical and internal delivery systems [20, 21] [22] [23].

Urease is widely used as analytical tool, for urea content analysis in blood, urine, alcoholic beverages, natural water and environmental wastewaters. Moreover it has been employed for removal of urea from artificial kidney dialyzates [24]. It has also been utilized for production of ammonia or carbon dioxide through urea hydrolysis. The use of stabilized urease formulations could be used as the means of more sophisticated organic synthesis catalyst:

Conventional syntheses of industrially important reagents such as dimethyl carbonate, ethylene carbonate and carbodihydrazide are challenged by factors such as low efficiency due to side reactions, mandatory use of toxic starting materials, high energy input, and inconvenient reaction conditions [19]. In view of the strategic importance of such compounds, alternative production methods boasting higher productivity and lower cost remain a subject of much interest. In theory, urease could prompt formation of the above desired products by enforcing reaction between the inexpensive substrate urea, and a non-water nucleophile such as methanol, ethylene glycol or hydrazine.

Pepsin is conventionally used in food and feed industries, in the processing of meat, fish, and milk and vegetable proteins (in the production of non-dairy foods). It also has wide applications

in leather industry, for removal of residual hair and tissue. They are employed for research and biomedical purposes, as the means of antibody cleavage and within formulation of digestive aids [25]. Furthermore, pepsin esterase activity, of stabilized immobilized formulations, could be used as organic synthesis catalyst.

CHAPTER 2 Urease Cross Linked Enzyme Aggregates (CLEA) and Nano Cross Linked Enzyme Aggregates (nano CLEA)

2.1 Introduction

Ureases (urea amidohydrolases, EC 3.5.1.5), whose catalytic function is to hydrolyse urea into carbonic acid and ammonia as final products and which are widely found in nature, are a group of highly proficient enzymes [26]. Ureases are produced from bacteria, fungi, yeast and plant [27]. As a primary function, ureases allow plant and bacteria to utilize urea in a proper way and also have a crucial role in nitrogen's metabolism of nature [28]. In 1926, the first crystal structure of urease was obtained from Jack bean (Canavalia ensiformis; JBU) [29] and this work gained a Nobel Prize in Chemistry in 1946. In Sumner's work, two different aspects have been well emphasized; the proof about the proteinaceous nature of enzyme and the crystallization ability of proteins. Urea, the substrate of urease, has also had a historical significance as being the first organic compound synthesized in 1828 [30].

There are some structural differences between ureases produced from plants and bacteria. Plant ureases are made up of single-chain polypeptide whereas bacterial ureases are made up of two or three polypeptides designated as α, β, and γ. In here, we have worked on JBU plant urease. It has been described in 3D structure of JBU that there are found two Ni ions separated by 3.7 Å [27]. Balasubramanian et al. described Ni binding in active site of JBU such that His519 , His545 and Lys490 residues liganded to N1 and His407, His409, Asp 633 and Lys490 residues liganded to Ni2[27]. As shown in Figure 2-1 [27], Lys490 residue is carbamylated and acted as to form a bridge between two Ni residues[27]. As described in activation mechanisms of other enzymes [31], there has been found a mobile flap in 3D structure of JBU. This mobile flap, existed between Met590 and His607 as a TIM-barrel, covers the active site of JBU and directly controls the entrance of substrate and the release of products [27]. Upon the changes in 3D conformations of this mobile flap, the active site of JBU becomes accessible and this change has been associated with the chemical modification and rearrangement of some residues, which can be accounted as a part of activation mechanism of JBU. It has been reported that Cys592, located in a mobile flap of

JBU, is well conserved among many ureases [32] and is one of three Cys residues in JBU, which underwent a chemical modification to alter enzymatic activity. It has been reported for JBU that 36 Cys residues have been found but only 3 of 36 (Cys59, Cys207 & Cys592) have undergone chemical modification that triggers the enzymatic activity.

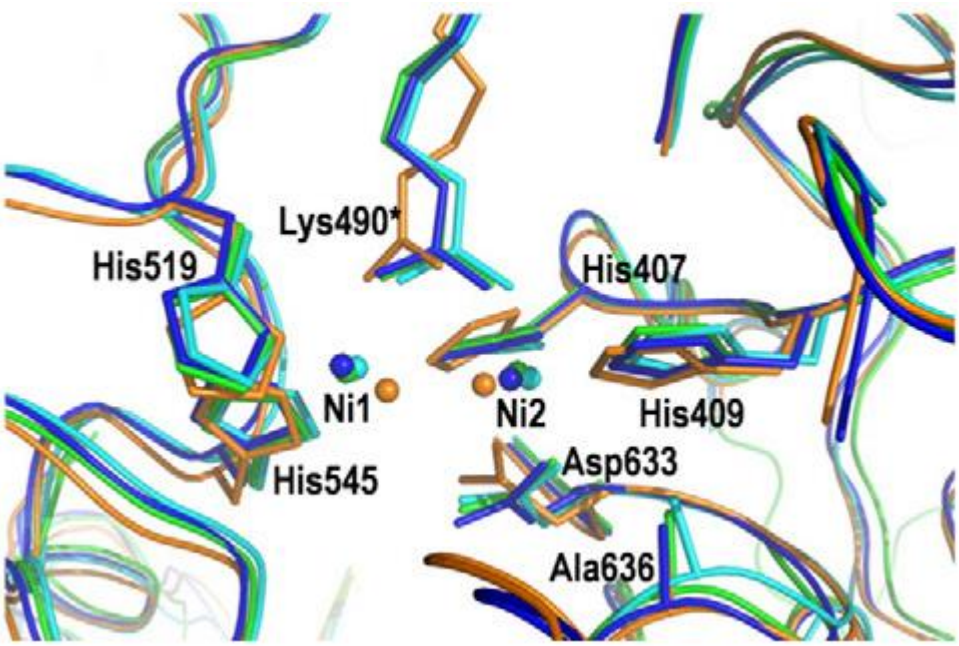


Figure 2-1 Active site of JBU (Jack Bean Urease) [27]

Up to now, two different activation mechanism have been proposed in literature for urease activity. First of all, the activation mechanism of urease has been proposed as the carbonyl oxygen atom of urea bind to Ni1 in active site of urease and it triggers the closed conformation of mobile flap. Then, the Ni2-bound to –OH group acted as a nucleophile to attack carbonyl carbon atom of urea, already polarized through coordination with Ni1. Upon formation and coordination of tetrahedral intermediate in active site, His320 acts as a general acid and leads to release of ammonia[33]. Benini et al. proposed another activation mechanism for ureases that urea binds Ni1 through bidentate manner with its carbonyl oxygen and immediately one of the amino group, bound to Ni2, replaces with tree water moieties and only the bridging hydroxide is left [34]. Upon

the attack of this hydroxide toward urea, the tetrahedral transition state is formed and it leads to formation of ammonia and carbamate.

Urease immobilization serves a challenging way of synthesis due to the restrictions on the active site shown in Figure 2-1. The surface residue numbers are respectively listed like; Lys: 37, Asp: 36 and Glu: 38. Furthermore, the total volume, total surface area and total solvent accessibility are listed respectively; 100073.0, 31071.8 and 32501.9 Å.

Even though there is a significant number of Lys groups present on the surface of urease both aggregation and crosslinking prove highly challenging. Urease is a moderately water soluble protein (up to 50 mg/ml). Furthermore, as can be observed from the surface structure majority of lysil residues are juxtaposed to carboxyl acid side chain baring amino acids. It can be said that urease is neither extensively hydrophilic nor hydrophobic; therefore the precipitation through depletion of available water surroundings is highly inefficiently. Furthermore, the challenged crosslinking could also be explained in the similar fashion, in terms that majority of the surface amine residues are not available due to intra molecular salt bridge interactions.

In this chapter, the production of the first urease (JBU) CLEAs is described through a modified aggregation procedure. Moreover, urease crosslinked enzyme lyophylizates (CLEL) assisted by incorporation with albumin are synthesized in order to further overcome difficulties related to urease processing.

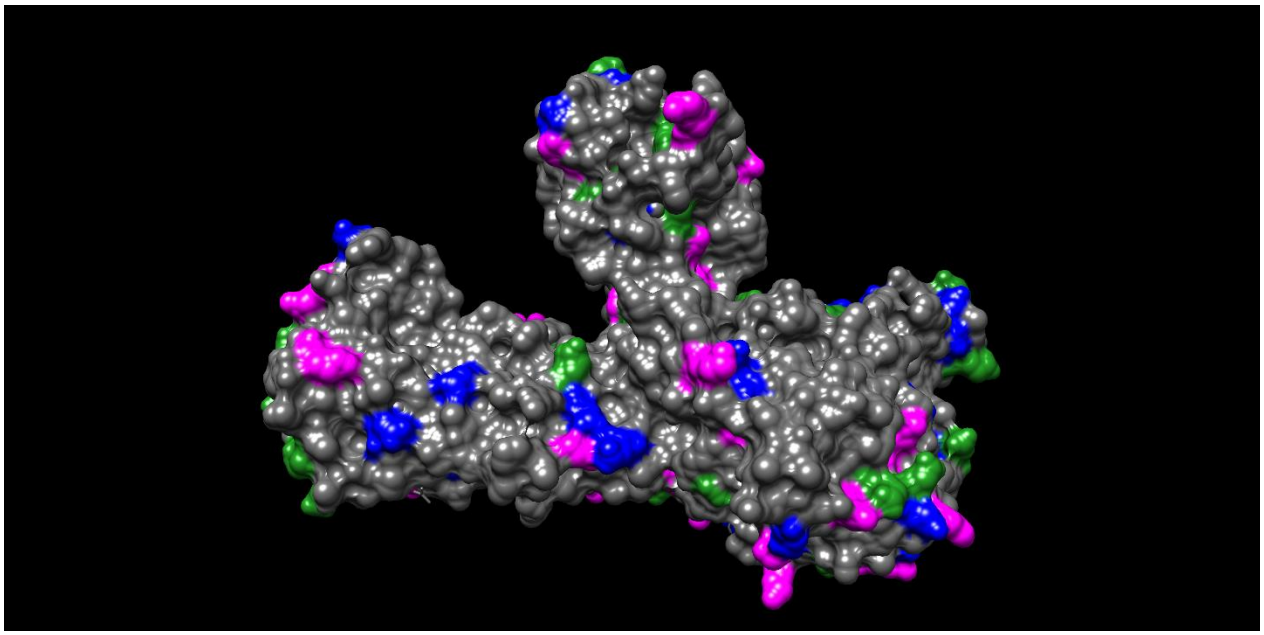


Figure 2-2 pdb structure of urease (pdb code: 3la4); Lys: Magenta, Asp: Blue, Glu: Green [27]

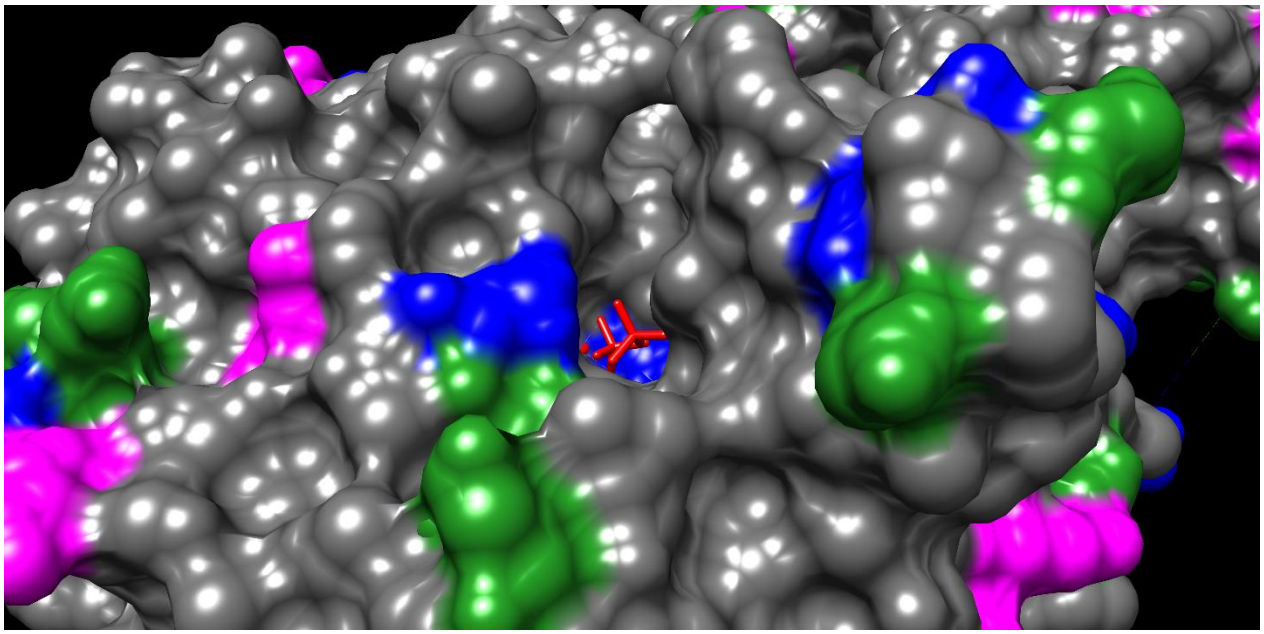
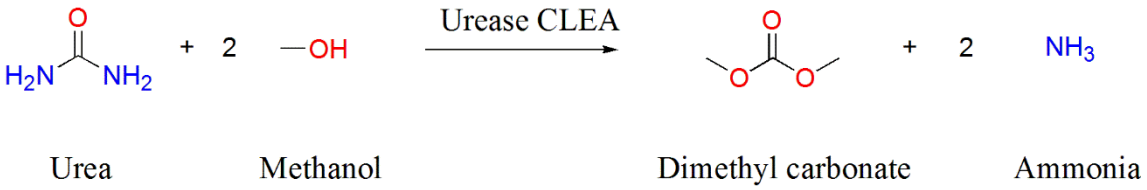


Figure 2-3 Presentation of the active site of urease (3D structure) (pdb code: 3la4); Lys: Magenta, Asp: Blue, Glu: Green, active site residues: Red [27]

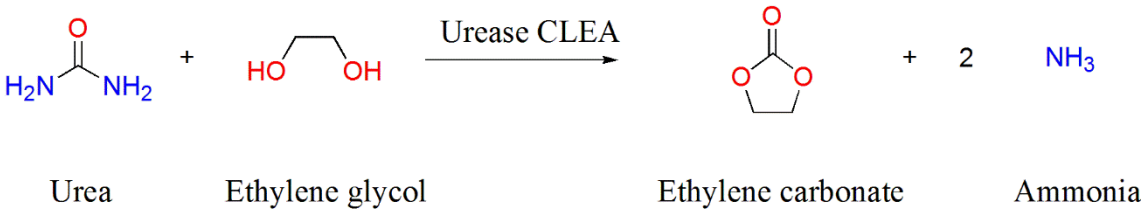
As mentioned in Chapter 1, there are several ways to immobilize enzymes for obtaining enhanced enzymatic activity and stability. Cross-linked enzyme/protein nanoaggregates have been produced in-house by a top-down methodology. In order to prepare the crosslinked nanoaggregates, the enzyme is normally subjected to crash-precipitation (via either salting out or antisolvent addition methods), crosslinking, and nanonization by hydrodynamic shear. Additives such as grinding aids, lyoprotectants, and cryoprotectants are introduced to facilitate the nanonization step and to promote optimal activity. This top-down nanonization approach is unique in the preparation of crosslinked enzyme nanoparticles, and it has been observed to prompt increased stability and activity in aqueous and non-aqueous media [35] [16].

Furthermore, cross linked urease lyophylizates were used in several reactions of urea as a way of catalyst. Reactions of urea with different reagents end up with significant chemicals like dimethyl carbonate, ethylene carbonate and carbodihydrazide. Accompanied by catalysts, yields of these reactions were not sufficient and also some drawbacks like difficulty of handling, expensiveness and toxic material exposure were faced [19]. Especially for synthesizing dimethyl carbonate (DMC) which is referred to as a green product, different ways to produce has drawn much attention in the previous years. DMC can be used as a substitute for chemicals such as phosgene for carbonylation processes and dimethyl sulfate (DMS) or methyl chloride for methylation reactions [36].

Reaction I:



Reaction II:



Reaction III:

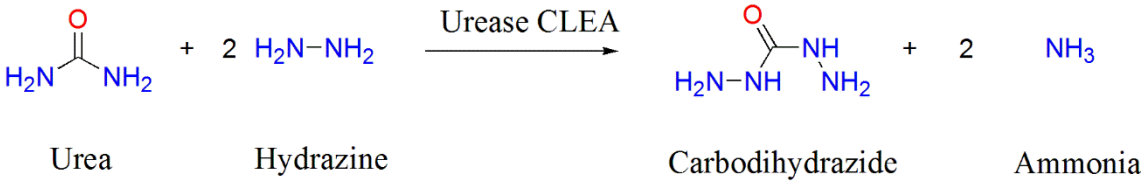


Figure 2-4 Targeted nucleophilic transformations of urea

2.2 Materials

Instrumentation:

Beckman Coulter centrifuge

Eppendorf centrifuge 5415D

Eppendorf centrifuge 5804 Eppendorf thermomixer® comfort

New Brunswick Scientific Innova 40 incubator shaker series

Homogenizer Heidolph silent crusher M

Christ brand ALPHA 1-2 LD plus laboratory scale freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany).

Leo G34-Supra 35 VP Scanning Electron Microscope

Malvern Instruments Zetasizer Nanoseries Nano ZS Dynamic Light Scatter

Shimadzu UV-3150 UV-VIS-NIR Spectrophotometer

Emitech K950X Carbon Vacuum Evaporator

Cressington Sputter Au/Pd Coater 108 auto

GC-MS QP2010 Ultra Shimadzu (column RTx-5MS guard, 30m, 10 um, 0.25 mm).

Chemical reagents and proteins:

Jack Bean Urease (EC 3.5.1.5) was obtained from Sigma-Aldrich. Urease from Canavalia ensiformis (Jack bean), Type IX, powder, 50,000-100,000 units/g solid

Bovine Serum Albumin was obtained from Sigma-Aldrich. Bovine Serum Albumin, heat shock fraction, pH 7, ≥98%

Glutaraldehyde, 25% aqueous solution, hydrazinium hydroxide (about 80% N_2H_5OH) and ninhydrin GR for analysis were obtained from Merck.

N,N'-dicyclohexylcarbodiimide was obtained from Sigma-Aldrich.

N-Hydroxysuccinimide, 98% was obtained from abcr GmbH&Co.

KGUrea was purchased from MP Biomedicals, LLC.

Ammonium sulphate was from Panreac Quimica S.A.U.

Sodium borohydride was obtained from Acros.

Ammonium carbonate was obtained from VWR.

Other reagents and solvents used were analytical or high performance liquid chromatography (HPLC) grade. All substances were directly withdrawn from their original stock and used without pre-treatment or further purification.

2.3 Methods

2.3.1 Urease CLPA Synthesis

2.3.1.1 Method A: Solution-phase crosslink-assisted aggregation & CLEA formation

Urease powder (10-50 mg/ml) was dissolved by mild agitation in phosphate buffer (100 mM, pH 7.4, 4 ˚C) and albumin powder (0-100 mg/ml) was subsequently added. The proteins in solution were pre-stabilized by addition of trace amount crosslinker directly into solution (10-40 µl, 25wt% , pH 5 / 20-80 µl, 12.5wt%, pH 9.2 glutaraldehyde or 10 µl 4 mg/ml aqueous N,N'-dicyclohexylcarbodiimide solution) and brief incubation (3 min, 4 ^{o}C). Mixture was precipitated thereafter by direct addition into crosslinking reagent containing solution of ammonium sulphate (4M; 4 ^{o}C) with continual stirring; With good stirring, protein solution was dropped into aggregation medium containing glutaraldehyde (100-400 µl, 25wt%, pH 5 / 200-800 µl, 12.5wt%, pH 9.2) or aqueous N,N'-dicyclohexylcarbodiimide solution (100 µl 4 mg/ml), and the main crosslinking reaction step was subsequently permitted for 20 h (4 ^{o}C). The crosslinked material was recovered as a pellet following centrifugation (5 min, 10 krpm), the pellet was treated with a freshly made aqueous solution of sodium borohydride (1000 µl, 1mg/ml dH_2O, 30 min), centrifuged (10 krpm, 5 min), and twice reconstituted (1000 µl, RT, 5 min) and centrifuged (10 krpm, 5 min) in distilled water to remove traces of reagent. The wet pellet was dried under vacuum (RT, 12 h).

Crosslinker reagents and protein composition variants for solution-phase crosslink-assisted method are summarized in the table below:

Table 2-1 Enzyme concentration, crosslinker and aggregation medium information for CLEA formation via solution-phase crosslink-assisted aggregation method

Enzyme concentration code	**E1**	**E2**	**E3**	**E4**
Enzyme concentration /ml	50 mg urease	50 mg urease 50 mg albumin	10 mg urease 100 mg albumin	25 mg urease 100 mg albumin
Aggregation medium code	**A1**	**A2**		
Aggregation/crosslinking medium	4M ammonium sulfate	1,4-dioxane		
Crosslinker code	**C1**	**C2**	**C3**	**C4**
Crosslinkers	Glutaraldehyde 25% pH 5	Glutaraldehyde 12.5% pH 9.2	N,N'-dicyclohexylcarbodiimide/ N,N'-dicyclohexylcarbodiimide	N,N'-dicyclohexylcarbodiimide/ Glutaraldehyde 25% pH 5

2.3.1.2 Method B: Lyophilization-mediated aggregation & CLEL formation

Starting solution was prepared with urease powder (20-50 mg/ml) and albumin (0-100 mg/ml) in phosphate buffer (100 mM, pH 7.4, 4 ^{o}C). The solution was transferred into 2mL capacity Eppendorf tubes, and the tube rack was submerged in liquid nitrogen followed by lyophilization (24h). After the lyophilization procedure, the lyophilizates were dropped into crosslinker-precipitant mixture (25% glutaraldehyde pH 5 / 12.5% glutaraldehyde pH 9.5 – dioxane/acetone/isopropanol/4M ammonium sulphate). Except for reactions performed in dioxane (4h, RT, 200 rpm), all the reactions took place under 4 ^{o}C (20 h, 500 rpm). The crosslinked material was recovered as a pellet following centrifugation (5 min, 10 krpm), the pellet was treated with a freshly made aqueous solution of sodium borohydride (1000 μl, 1mg/ml dH_2O, 30 min), centrifuged (10 krpm, 5 min), and twice reconstituted (1000 μl, RT, 5 min) and centrifuged (10 krpm, 5 min) in distilled water to remove traces of reagent. The wet pellet was dried under vacuum (RT, 12 h).

Crosslinker reagents and protein composition variants for solution-phase crosslink-assisted method are summarized in the table below:

Table 2-2 Enzyme concentration, crosslinker and aggregation medium information for CLEA formation via lyophilization method

Enzyme solution code	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**
Concentration /ml	20 mg urease	20 mg urease 20 mg albumin	20 mg urease 60 mg albumin	20 mg urease 100 mg albumin	20 mg urease 20 mg albumin 50 mg sucrose	20 mg urease 60 mg albumin 50 mg sucrose	20 mg urease 100 mg albumin 50 mg sucrose
Crosslinking medium code	**M1**	**M2**	**M3**	**M4**			
Crosslinking medium	4M ammonium sulfate	1,4-dioxane	Acetone	Isopropanol			
Crosslinker code	**C1**	**C2**					
Crosslinker	Glutaraldehyde 25% pH 5	Glutaraldehyde 12.5% pH 9.2					

Crosslinkers

Glutaraldehyde

Glutaraldehyde was applied at concentrations of 2.12 x 10^{-5} mol per mg protein (dry weight equivalent). Glutaraldehyde is typically stored and sold at slightly acidic pH values, which serves to reduce its optimal reactivity. In this work, commercial glutaraldehyde stocks (25wt%, pH 5) were directly used without pH adjustment. Alternatively, water-diluted stocks (12.5wt%, adjusted to pH 9.2 using 0.1M sodium carbonate buffer and pH 7.4 using 0.1M sodium phosphate buffer) were used.

N,N'-dicyclohexylcarbodiimide

N,N'-dicyclohexylcarbodiimide (DCC) was applied at $1.9x10^{-5}$ mol concentrations per mg protein (dry weight equivalent).

Figure 2-5 Representation of lyophilization method in freeze-drier

2.3.1.3 Organic Reactions of Urea with Urease CLEL

For preparing precursor solutions, 75 mg urea/1 ml methanol and 108 mg urea/1 ml ethylene glycol were dissolved under heat and sonication (30 min, 50 ^{o}C).

0.622 ml hydrazine was first dissolved in 20 mL methanol and 20 mL ddH_2O giving hydrazine-methanol and hydrazine-H_2O solutions. Afterwards, 120 mg urea/1 ml hydrazine-methanol, 120 mg urea/1 ml hydrazine- H_2O were dissolved.

3.5 mg of CLEA (E3.M1.C2 sample, See Table 2-2) was placed into 2 mL Eppendorf tubes with 1 mL of 75 mg/ml methanol/urea, 108 mg/ml ethylene glycol/urea and 120 mg/ml

hydrazine/methanol/urea – hydrazine/ H_2O/urea solutions for the synthesis of dimethyl carbonate, ethylene carbonate and carbodihydrazide (4h, 25/50 ^oC).

The molar ratios of the reactions can be seen on the table below:

Table 2-3 Molar ratios of urea reactions

		Mol	**Molar ratio**
Reaction I	Urea	0.012	20
	Methanol	0.25	
Reaction II	Urea	0.02	10
	Ethylene glycol	0.18	
Reaction III	Urea	0.02	1
	Hydrazine	0.02	

2.3.2 Nano CLEA Generation

Nanonization was achieved via hydrodynamic shear application using homogenizer (Heidolph silent crusher M) with varying processing time and shear conditions. In a typical run, CLEA (2.5 mg) was dispersed in 1 ml 30% aqueous glycerol solution or 100% ethanol and nanonization was performed using different instrumental settings and times (10-21 krpm; 30-60 min). Given the thermal stability of CLEAs, no elaborate steps were taken to avoid incidental heating of the dispersion during nanonization.

The labeled nano-CLPA samples were transferred into 1.5 ml eppendorf tubes with the dialysis membrane replacing the top of the tube cap, tubes were further secured with parafilm tape to avoid any leakage. All samples prepared as described were dialyzed against pH 7.4 phophate buffer, with constant agitation, for the period of 6 hours, repeated 4 times. (Snake Skin® Dialysis Tubing, 3.5K MWCO, 35 mm dry I.D, 35 feet was obtained from Thermo Scientific).

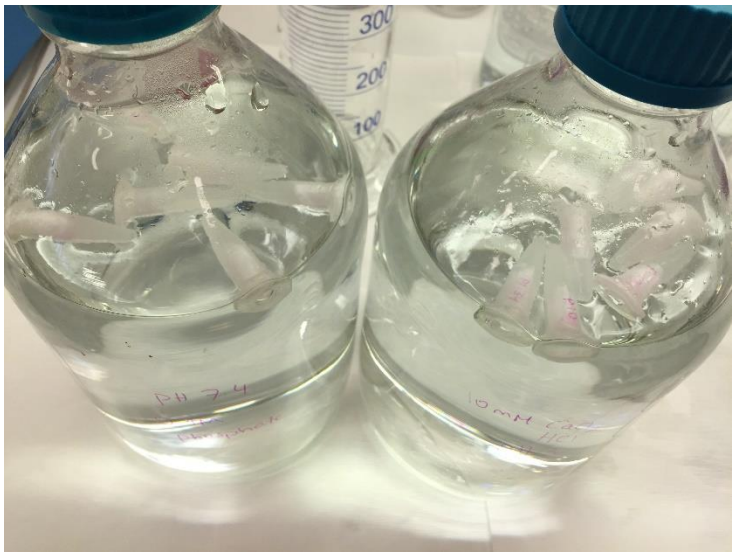


Figure 2-6 Representation of dialysis method in 1.5 ml Eppendorf tubes

2.3.3 Characterization of CLPA and Nano CLPA

The instrumental analysis was performed via Dynamic Light Scattering and Scanning Electron Microscopy.

2.3.3.1 Dynamic Light Scattering Measurements

The sample was diluted 10 fold in medium corresponding to their homogenization conditions. DLS data was collected on samples equilibrated at 25 ºC in 2ml disposable cuvettes, as a result of 3 consecutive scans, Malvern Zetasizer NANO ZS. Absorption of each sample was measured at 633 nm and included in DLS measurement protocol. Particle refractive index of 1.5 was assumed for all CLPA samples and refractive index of corresponding medium was included in the protocol. Data was analyzed using protein analysis model, Malvern Zetasizer software.

2.3.3.2 Scanning Electron Microscopy Analysis

CLPA powder was subjected to treatment in a Cressington Sputter Au/Pd Coater. An approximate coating thickness of 2-3nm was targeted. The processed samples were loaded into

the vacuum chamber of a ZEISS brand LEO SUPRA 35VP model SEM with GEMINI column. An electron gun voltage of 2kV was employed throughout the analyses.

2.3.3.3 Urease Catalytic Activity Assay Protocol

For the determination of the urease activity, a colorimetric assay based on ninhydrin color yield was applied to the samples to detect the free amines. A 3h activity assay reaction at room temperature took place for 2 mg/ml crosslinked protein nanoaggregates and isopropanolic ninhydrin solution (50 µl; 1 wt%) was used as the reagent for the supernatant of the samples (50 µl) and after 1h incubation at 70 ^{o}C, the UV-Vis spectrophotometric measurements (595 nm) were done for all the samples. Relative activity (%) was compared by assessing the urease mass fraction within each coCLEA against an equal mass of freely soluble native urease. The native urease was arbitrarily assigned a value of 100%.

2.3.3.4 Colorimetric Analysis of Urea Conversion Efficiency for Urea Reactions

For the determination of the urea conversion rate of the reactions, a colorimetric assay based on ninhydrin colour yield was applied to the samples to detect the free amines. Isopropanolic ninhydrin solution (50 µl; 1 wt%) was used as the reagent for the supernatant of the reaction samples (50 µl) and after 1h incubation at 70 ^{o}C, the UV-Vis spectrophotometric measurements (595 nm for methanol and ethylene glycol, 470 nm for hydrazine) were done for all the samples. Relative activity (%) was compared by assessing each sample against methanol/urea, ethylene glycol/urea and hydrazine/methanol/urea - hydrazine/H_2O/urea blank solutions. The absorption values were converted into concentration values using ammonia calibration curve.

2.3.3.5 Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis for Urea Reactions

In here, just the dimethyl carbonate reaction product was subjected to gas chromatography-mass spectroscopy analysis. The supernatant of the reaction sample was diluted 1:10 in methanol. The oven temperature program was: initial temperature 27 °C, hold for 5 minutes, ramp at 10 °C/min to 240 °C, hold for 5 minutes. The injector transfer line temperature was set to 150 °C. Measurements were performed in split–split mode (split ratio 10:1) using helium as the carrier

gas (flow rate 0.70 mL/min). For the mass spectra, solvent cut time was 2.5 minutes. Ion source temperature was 200 °C and the interface temperature was 250 °C.

2.4 Results and Discussion

Initial attempts to crosslink urease via conventional CLEA method were met with difficulties which were due to a protein-related difficulty in efficiently precipitating and possibly crosslinking the precipitated urease. The problem of crosslinking in particular was presumed to be related to a high tendency to form relatively inert intra molecular ammonium carboxylate bridges as well as few notable attachment points (Figure 2-1), as implied by the pdb structure of urease. Consequently, variants of established methods to prepare urease CLEAs were devised in hopes to bypass this impasse. Amongst the attempted methods, the most promising results were obtained via a solution-phase crosslink-assisted coaggregation method, and co-lyophilization method both accompanied by co-precipitation with albumin. With the first method in particular, urease and the readily-precipatatable albumin were initially allowed to crosslink in aqueous solution by introducing traces of glutaraldehyde or more surprisingly traces of the organic-soluble N,N'-dicyclohexylcarbodiimide. Both reagents are known to link reactive functional groups, and the nature of their chemistry substantially differs [6]. By way of this unorthodox strategy, an easily or readily co-precipatatable urease-albumin derivative was afforded, which could then be crosslinked via normal CLEA methods and subsequently transformed into nanoparticles via a top-down method as specified by Taralp [35]. In the second method, urease and albumin as carrier protein were co-lyophilized and the resultant powder was rapidly dispersed into different aqueous phase crosslinker media comprising of glutaraldehyde or possibly glutaraldehyde and an additional crosslinking reagent. The insoluble powder afforded could once again be retrieved via centrifugation and nanonized. The advantage of the second method was based on the premise that lyophilization would necessarily enforce a 100% solute-to-powder transition, hence bypassing any possibility of material loss. Hence by means of either method, urease was obtained in insoluble powder form of coaggregate together with albumin. The above work was also significant in the general sense that either method shows promise as an alternative to crosslink other proteins, which do not efficiently precipitate using established crash-precipitation (using anti-solvent and salting-out methods).

Herein the performance of desolubilized micron- and nanosized urease powders has been presented following crosslinking by each of the two methods. Subsequently, urease formulations were utilized as a hydroxyalkyl-de-amination and hydrazino-de-amination [37, 38], transforming urea into dimethylcarbonate, ethylenecarbonate and carbodihydrazide by selective addition of methanol, ethylene glycol, or hydrazine, respectively (Figure 2-4). It is hoped that further development of these methods will yield biologically optimized CLEAs from urease as well as other user-unfriendly proteins, opening a door to the routine preparation of industrially important chemical feedstocks.

2.4.1 Urease CLEA Synthesis

2.4.1.1 CLEA synthesis via solution phase crosslink assisted aggregation method

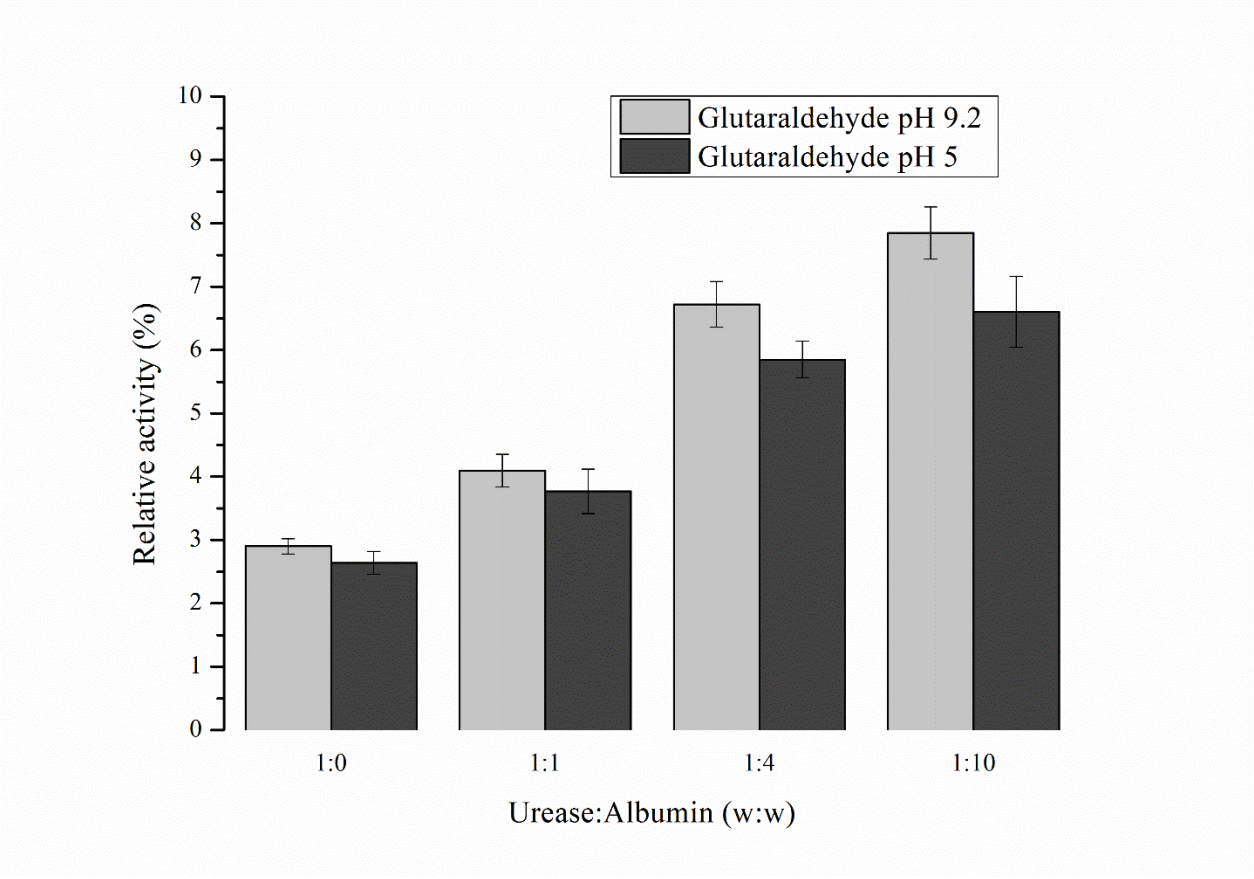


Figure 2-7 Effect of urease to albumin weight ratios and glutaraldehyde reagent pH on relative catalytic activities of urease CLEA. (Crash precipitation facilitated by saturated ammonium sulphate solution)

Figure 2-7 illustrates the activity of equal amounts of urease nano CLEAs coprecipitated in aqueous ammonium sulphate using different amounts of albumin as stabilizing additive. In all cases, activity noted was higher for alkaline crosslinking. Moreover, the absolute activity was seen to incrementally increase with the amount of albumin present. The root cause of the varied apparent bioactivity was not specifically investigated, but it is likely related to differences in spatial distribution and interaction between urease and albumin, allowing for better active site access, higher fraction of catalytically competent protein, and/or higher intrinsic catalytic efficiency with increasing albumin loadings. Amongst some potential factors, one contributor might have been an enhancement of the surface availability of urease with increasing albumin content. Another possibility was that albumin imparted an activating /protecting effect in the sense that larger amounts of albumin permitted urease to retain higher activity, via any number of secondary effects such as better retention of native structure. It is also possible that the course chemical crosslinking could follow an albumin-loading dependency, leading to variations in specific site reactions along the surface of urease, as well as varied protein conformation and rigidity. Since nano-CLEAs were shown in-house to not have diffusional limitations in the case of small substrates, it follows to reason that a potential catalytic or conformation-protecting effect of BSA is at least the major contributor as opposed to differences in particle morphology and porosity, which would in turn directly influence mass transfer and active site accessibility by substrate.

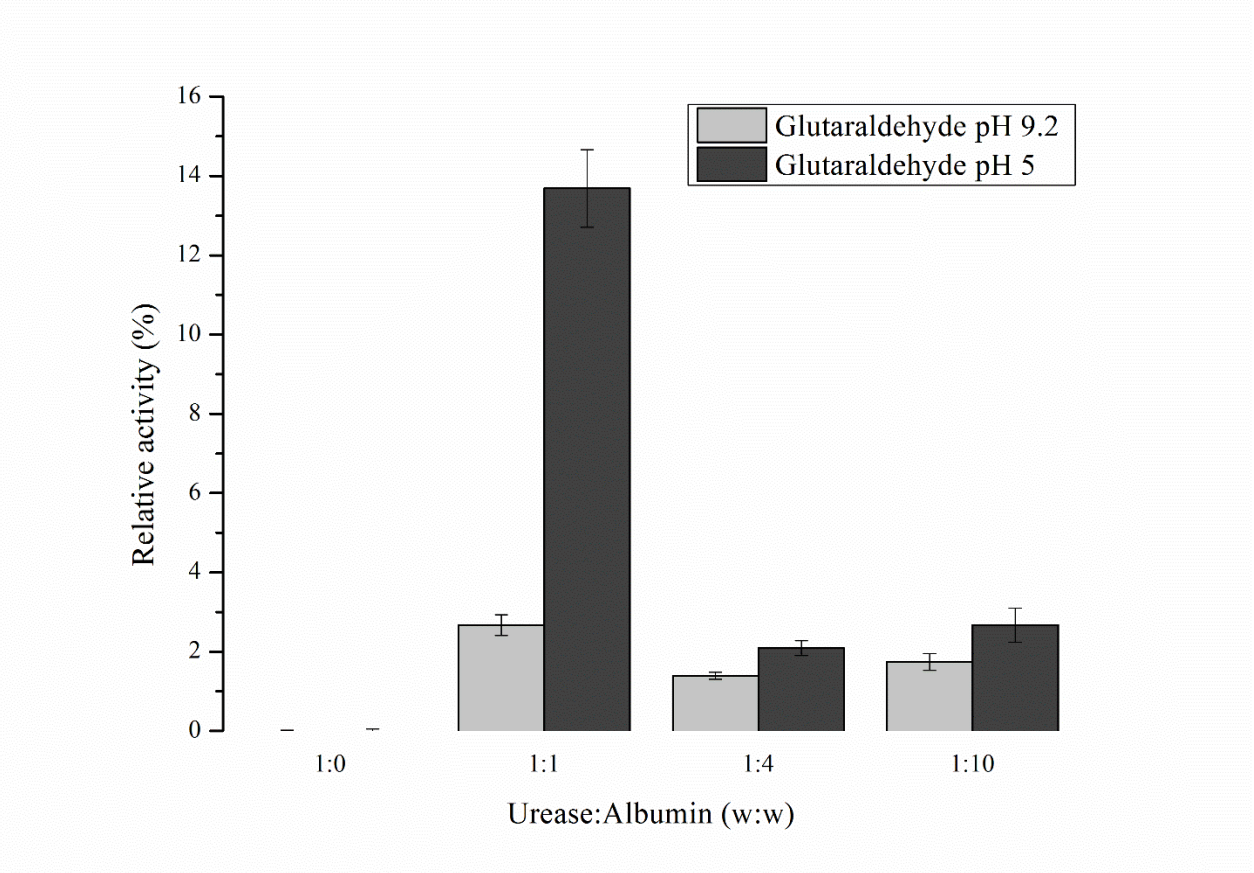


Figure 2-8 Effect of urease to albumin weight ratios and glutaraldehyde reagent pH on relative catalytic activities of urease CLEA. (Crash precipitation facilitated by 1,4-dioxane)

Figure 2-8 shows the relative activity (%) of same amounts of urease CLPAs precipitated in the solvent 1,4-dioxane with the help of changing amounts of carrier protein BSA. 1:1 weight ratio acidic co-precipitate gave around 14% activity whereas the others were unable to show some activity in the solvent 1,4-dioxane crash precipitation.

The relatively high bioactivity of 1:1 urease/albumin CLEAs would be consistent with the action of albumin carriers in promoting stability and bioactivity In keeping with this argument, larger loadings of albumin must have encapsulated the urease units to the point of precluding substrate access. This explanation is particularly suitable given the ability of 1,4-dioxane to prompt

structural rigidity. The precise reason is unclear why 1:1 urease/albumin crosslinked in acidic glutaraldehyde yielded 14% activity whereas the basic glutaraldehyde yielded near-zero activity.

However, difference of glutaraldehyde species formation in aqueous and organic media could be attributed to these results as compared to aqueous crosslinking conditions demonstrated on the previous graph. Therefore, these results could be related to differences in crosslink location, crosslink density, chemical inactivation, and conformational disruption.

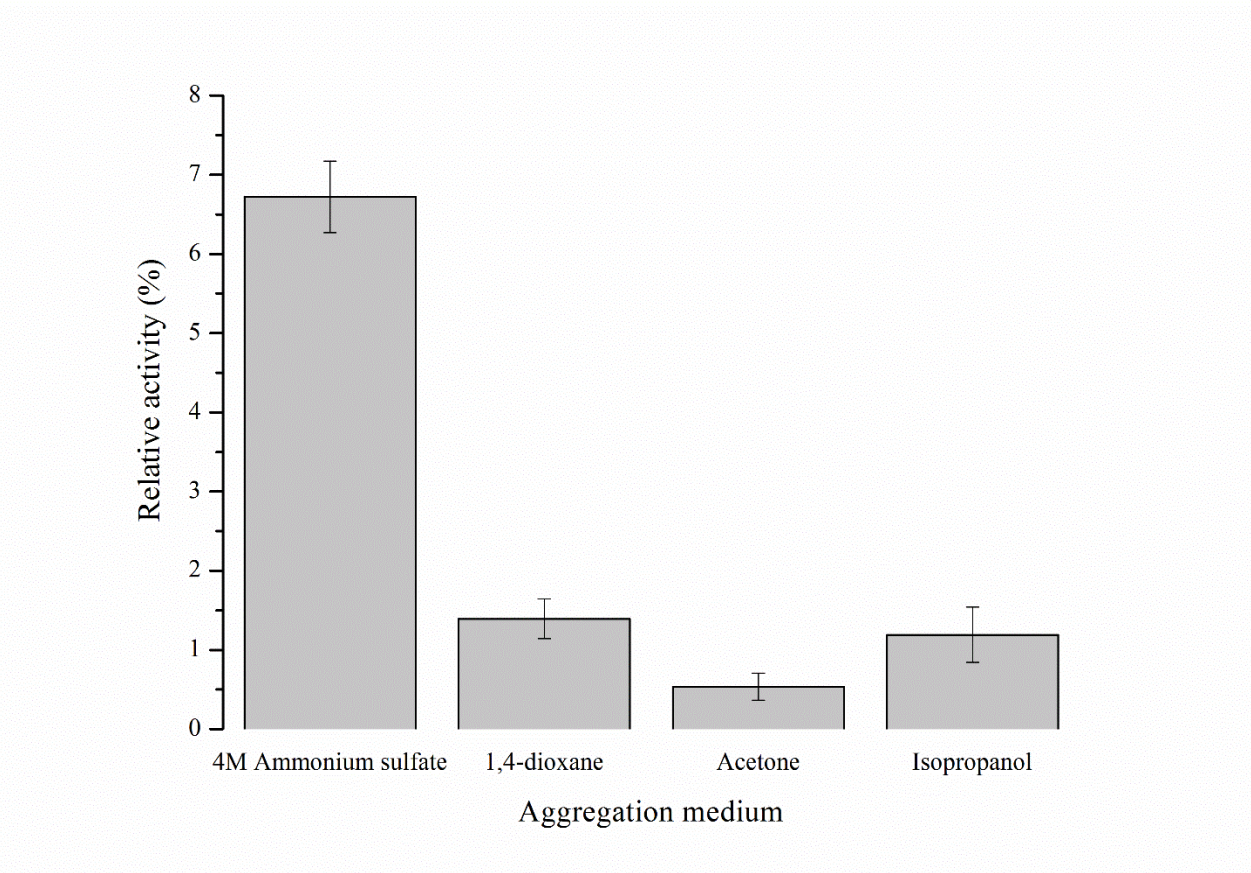


Figure 2-9 Effect of aggregation medium on relative catalytic activity of urease CLEA. (1:4 urease to albumin weight ratio, crosslinking facilitated by glutaraldehyde pH 9.2)

Figure 2-9 presents a graph of relative activity (%) changing via aggregation medium. For this assay, 1:5 (Urease:Albumin; w:w) sample crosslinked with basic glutaraldehyde was used. 4M

ammonium sulfate showed a significant difference on the activity as compared to other mediums (1,4-dioxane, acetone and isopropanol).

Partial aqueous-phase crosslinking prior to 1,4-dioxane precipitation served to confirm the veracity of the overall method, however, in light of challenges posed by the use of 1,4-dioxane as anti-solvent, the brunt of the work was continued using a more universal and well-established salting out agent. In particular, aqueous ammonium sulphate was selected.

1,4-dioxane was initially used, as it proved to be the only anti-solvent, which could near-quantitatively precipitate urease (not shown) as well as the initial solution phase pre-crosslinked urease. That being said, the urease CLEAs thus showed no activity. The situation was notably ameliorated by the equi-weight presence of albumin but no advantage was noted in proceeding to higher albumin/urease ratios, as has been shown above (Figure 2-8).

Work using ammonium sulphate proved noteworthy in comparison to initial trials using 1,4-dioxane. Given the more positive apparent activities, ammonium sulphate clearly allowed for a greater retention of catalytically competent sites or greater average intrinsic reactivity. The root cause is likely related to more dynamic and possibly looser aggregate formation in ammonium sulphate compared to 1,4-dioxane, which might have changed the course of crosslinking as well as imparted increased conformational flexibility of the ensuing structures and better active site accessibility.

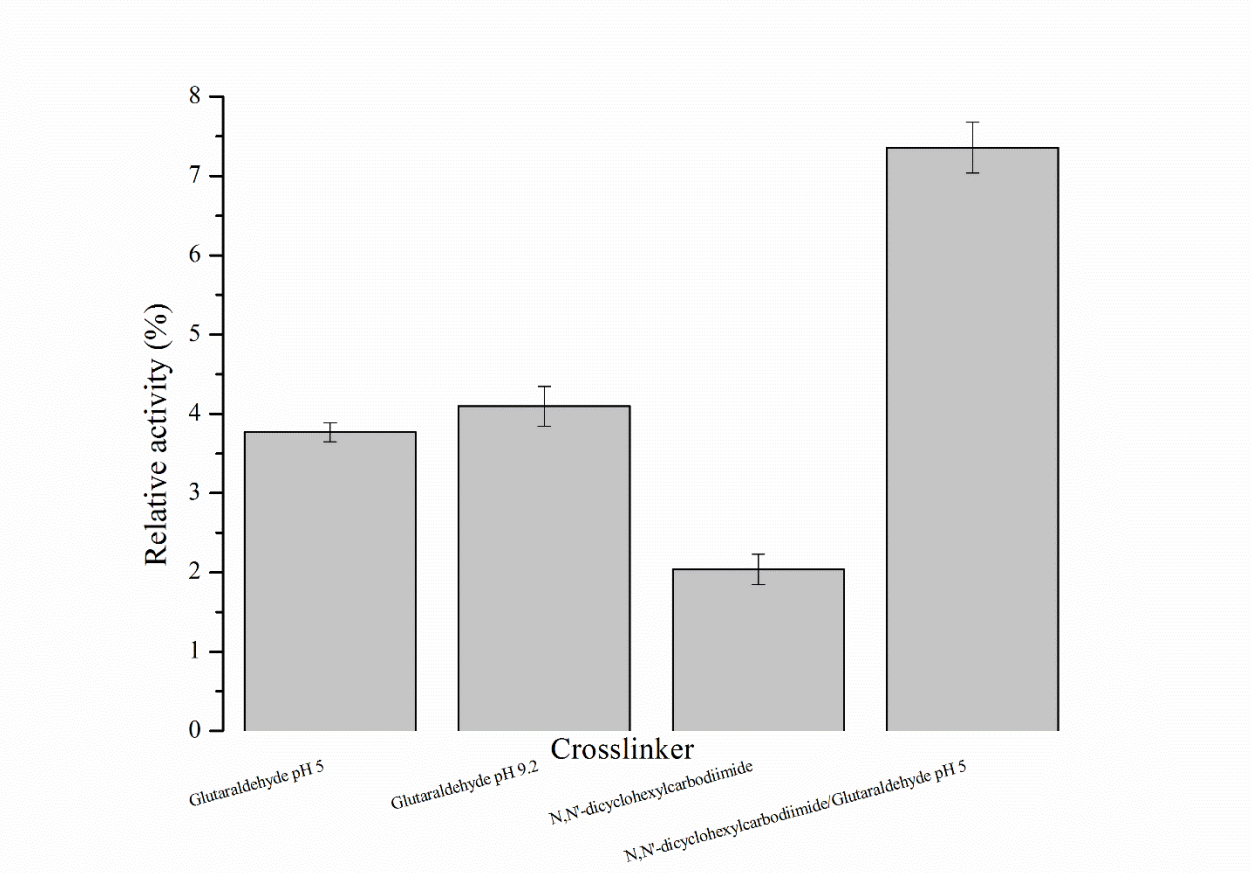


Figure 2-10 Effect of cross linking reagent on relative catalytic activity of urease CLEA. (1:1 urease to albumin weight ratio, crash precipitation facilitated by saturated ammonium sulphate solution)

Figure 2-10 illustrates changes of relative activity (%) as a function of different crosslinking media. In all cases, a 1:1 urease/albumin ratio was used prior to ammonium sulphate precipitation. As shown, the highest activity was observed for N,N'-dicyclohexylcarbodiimide/Acidic glutaraldehyde, with glutaraldehyde present in trace amounts.

Comparing the relative activities, the highest value belongs to the sample crosslinked with N,N'-carbodiimide/Acidic glutaraldehyde pair. N,N'-dicyclohexylcarbodiimide/N,N'-dicyclohexylcarbodiimide and acidic glutaraldehyde/acidic glutaraldehyde crosslinker pairs didn't show the same high activity. Neither did basic glutaraldehyde/basic glutaraldehyde pair.

This can only be explained by the trace amount of a "different" croslinker effect in the solution, activating a number of carboxyl residues and subsequent zero-length covalent bond formation with the juxtaposing amino- groups in addition to the covalent species formed in the main crosslinking step.

2.4.1.2 CLEL synthesis via lyophilization method

While the method described as "solution phase crosslink assisted aggregation" has facilitated formation of CLEA, unachievable through conventional procedure, the overall synthesis yields and resultant catalytic activity remained dramatically low. Results bellow present much improved efficiency in both catalytic activity and the overall yield, arising from substitution of aggregate formation with lyophylzation.

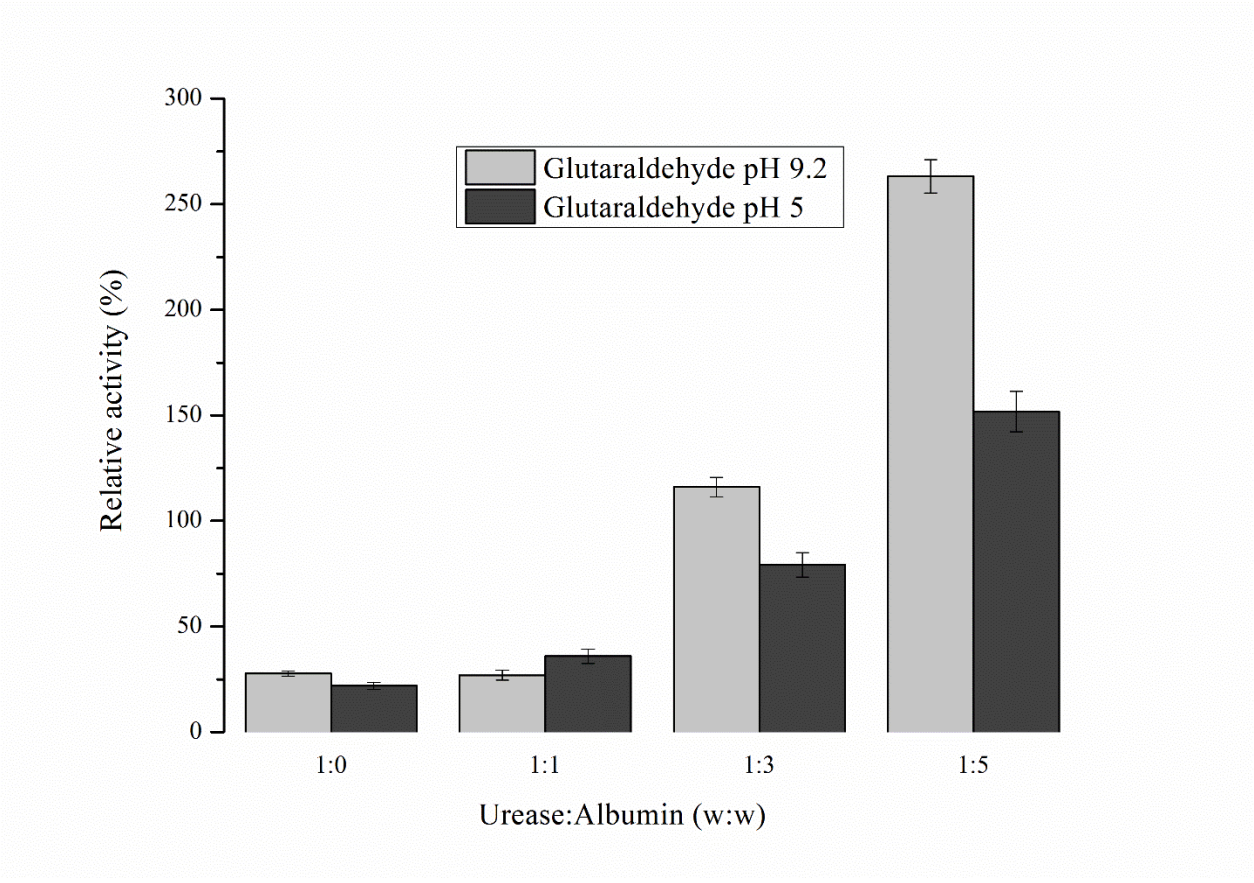


Figure 2-11 Effect of urease to albumin weight ratio and glutaraldehyde reagent pH on relative catalytic activity of urease CLEL. (Crosslinking medium- saturated ammonium sulphate solution)

Figure 2-11 presents the relative catalytic activity dependent on urease:albumin (w:w) ratio. The urease to albumin weight ratios are changing through 1:0, 1:1, 1:3 and 1:5. Moreover, there are two different pH values for the crosslinker glutaraldehyde (acidic and basic). The highest activity is assigned to 1:5 urease:albumin which was crosslinked with alkaline glutaraldehyde.

Herein two factors are assumed to significantly contribute to the results in Figure 2-11: Firstly, much like the results of solution phase crosslink assisted method have shown, albumin had an important influence on the activity results. This occurs due to the protective effect of albumin over urease on crosslinking. Comparing the 1:1 and 1:5 results, it is seen that 1:5 possesses higher catalytic yield. The second factor contributing to the results is that when basic glutaraldehyde was preferred to acidic one for the crosslinking step, a notable change on the relative activity was observed. The observed effect could be attributed to formation of highly reactive polymeric glutaraldehyde species at basic pH in aqueous media, facilitating higher number of overall crosslinking degree and thereof enhancing the stabilization effect.

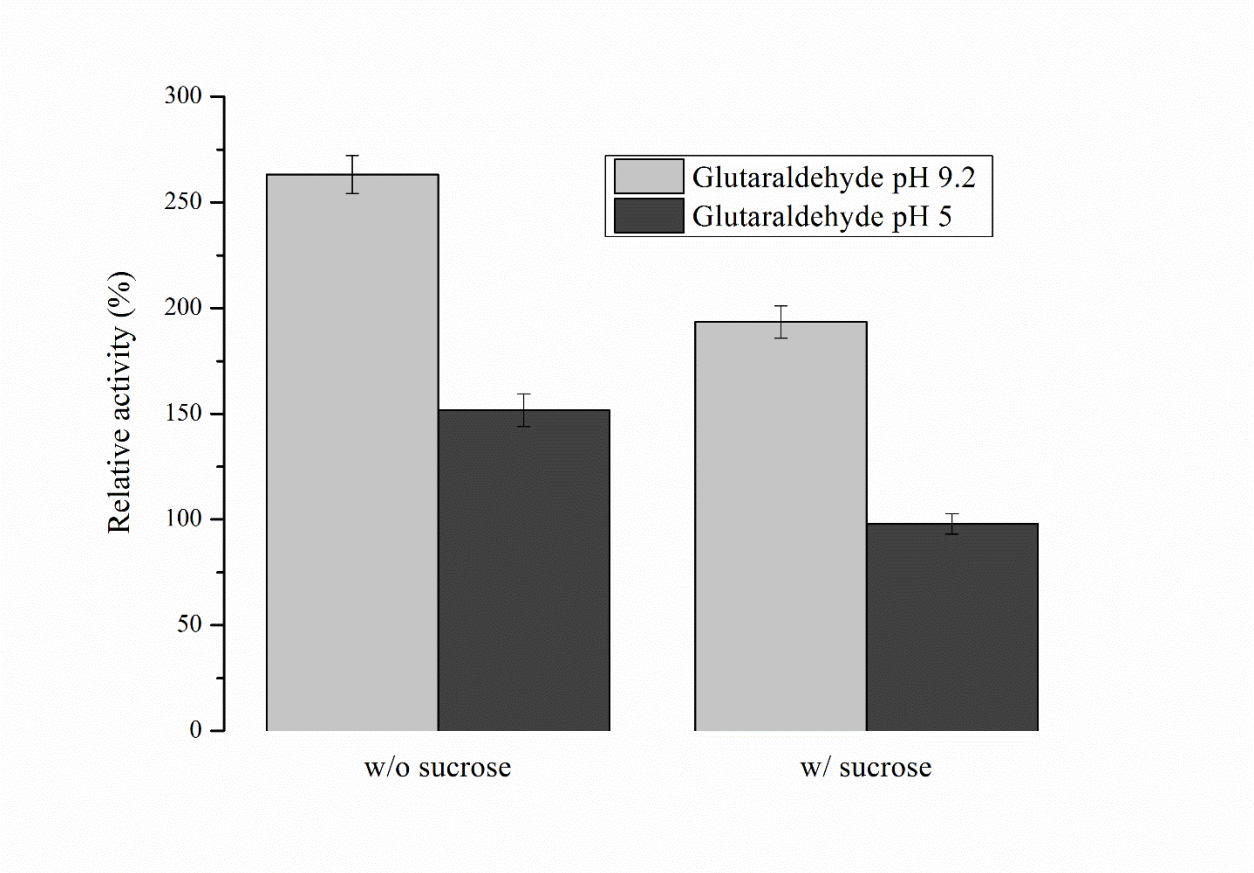


Figure 2-12 Effect of sucrose incorporation into co-lyophilizate composition and glutaraldehyde reagent pH on relative catalytic activity of urease CLEL. (1:5 urease to albumin weight ratio, crosslinking medium-saturated ammonium sulphate solution)

Figure 2-12 presents the relative catalytic activity change via sucrose addition and crosslinker pH change. The graph shows the effects on the urease:albumin, 1:5 (w:w) 4M ammonium sulphate CLEL preparation. As seen from the graph, sucrose addition, which initially was incorporated as a cryoprotectant, resulted in a decrease of the relative catalytic activity. Again, glutaraldehyde pH 9.2 results in higher catalytic activities compared to glutaraldehyde pH 5.

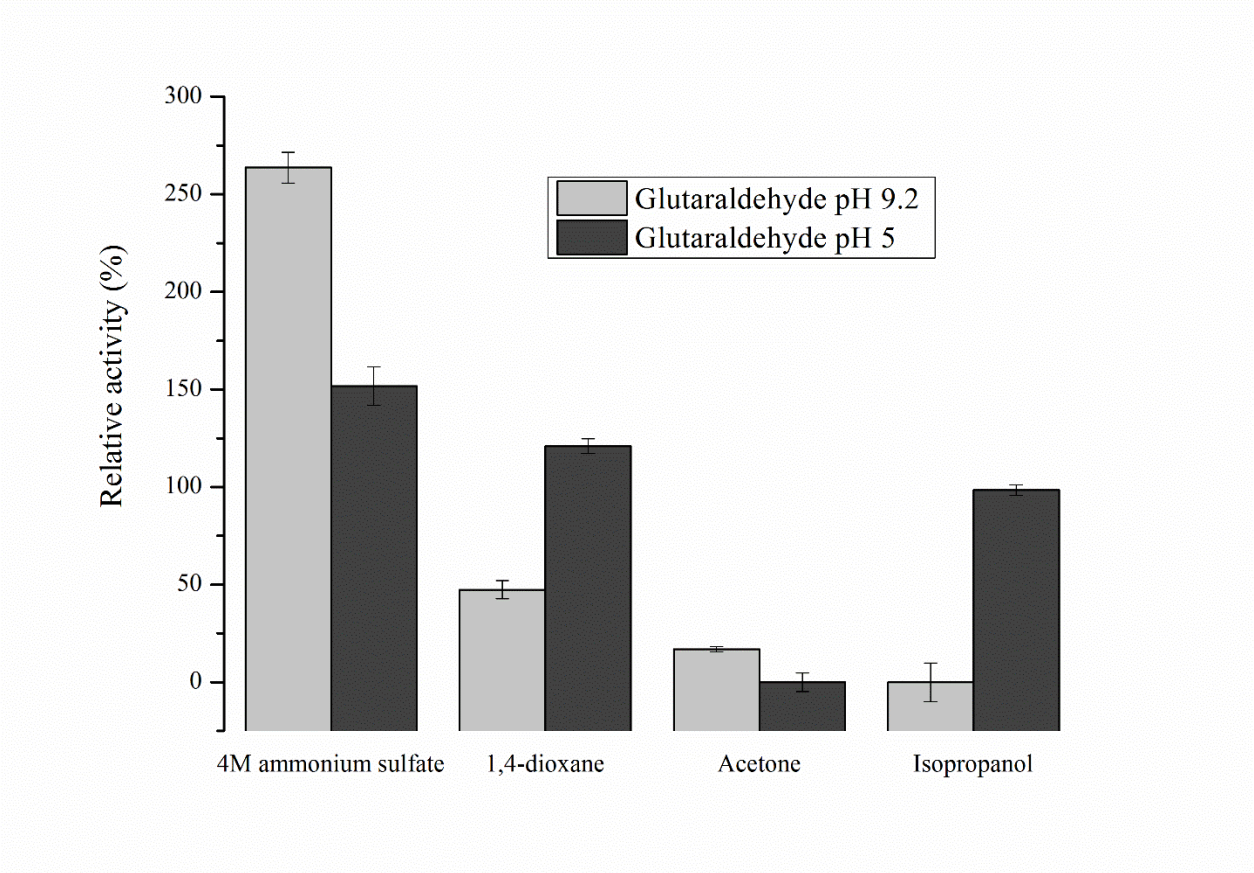


Figure 2-13 Effect of crosslinking medium and glutaraldehyde reagent pH on relative catalytic activity of urease CLEL. (1:5 urease to albumin weight ratio)

Fig 2-13 illustrates a graph of catalytic relative activity (%) changing via crosslinking medium. For this assay, 1:5 (Urease:Albumin; w:w) trials were conducted using crosslinking with both glutaraldehyde pH 5 and pH 9.2. As can be remembered from the solution phase crosslink assisted aggregation method catalytic relative activity graph (effect of aggregation medium), 4M ammonium sulphate was the medium that provides the highest relative activity (%) compared to the anti-solvents; 1,4-dioxane, acetone and isopropanol. Herein, the same effect can be observed from the graph. 4M ammonium sulphate, with the effect of the crosslinker glutaraldehyde pH 9.2, gives the highest activity. The results could similarly be rationalized, through further tightening of lyophylizate materials in antisolvent medium and therefore restriction of resultant crosslink material flexibility.

2.4.1.3 Stability of the Cross Linked Enzyme Lyophilizates

Upon catalytic activity measurements on previously synthesized cross linked urease lyophilizates (up to 6 months), no loss of activity was observed, affectively underlining shelf life stability of the developed formulations.

2.4.2 Nano Urease CLPL Synthesis

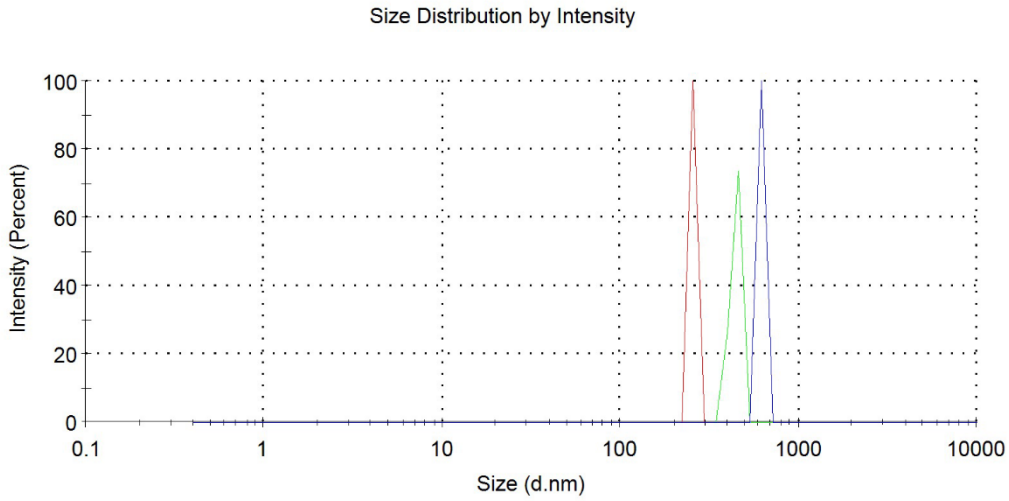


Figure 2-14 DLS result of nano crosslinked urease lyophilizate homogenized in absolute ethanol, at 21 krpm for 30 min (E3.M1.C1 – See Table 2-2)

Figure 2-14 presents a DLS measurement of supernatant (1krpm) of a urease CLEA suspended and homogenized in 100% ethanol. This data provided evidence of nanoparticle content generation upon nanonization procedure.

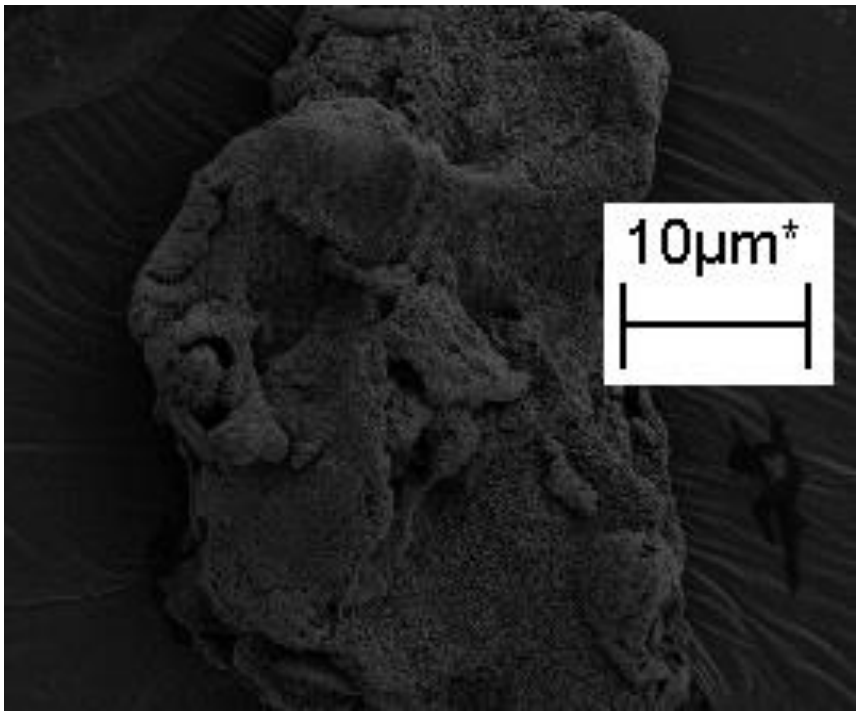


Figure 2-15 SEM imagery of crosslinked urease lyophilizate (CLEL), presenting the morphology of micro particle units; 2.00 KX, EHT = 2.00 kV, WD = 8 mm, Secondary electron detector

A general morphology of homogenization product is observe on Figure 2-15. SEM results are illustrated on Figure 2-16 presenting the morphology and approximate size order of heterogeneously nanosized cross linked urease lyophilizates and interior of the generated particles on Figure 2-17. The finely structured heterogeneouse size nano particles with the average size around 200 nm can be observed (Figure 2-16). The particles of similar morphology have been observed in the previous study (Zakharyuta, A., PhD Thesis, Nanosized Crosslinked Protein Aggregates (nano-CLPA)). Nevertheless, in the case of urease the majority of the material is of the appearence visualized on Figure 2-17. This morphology also reflects the interior of the finely structured particles demonstrated, thereof providing evidence of mechanical integrity loss of the major part of crosslinked material in the course of homogenization process.

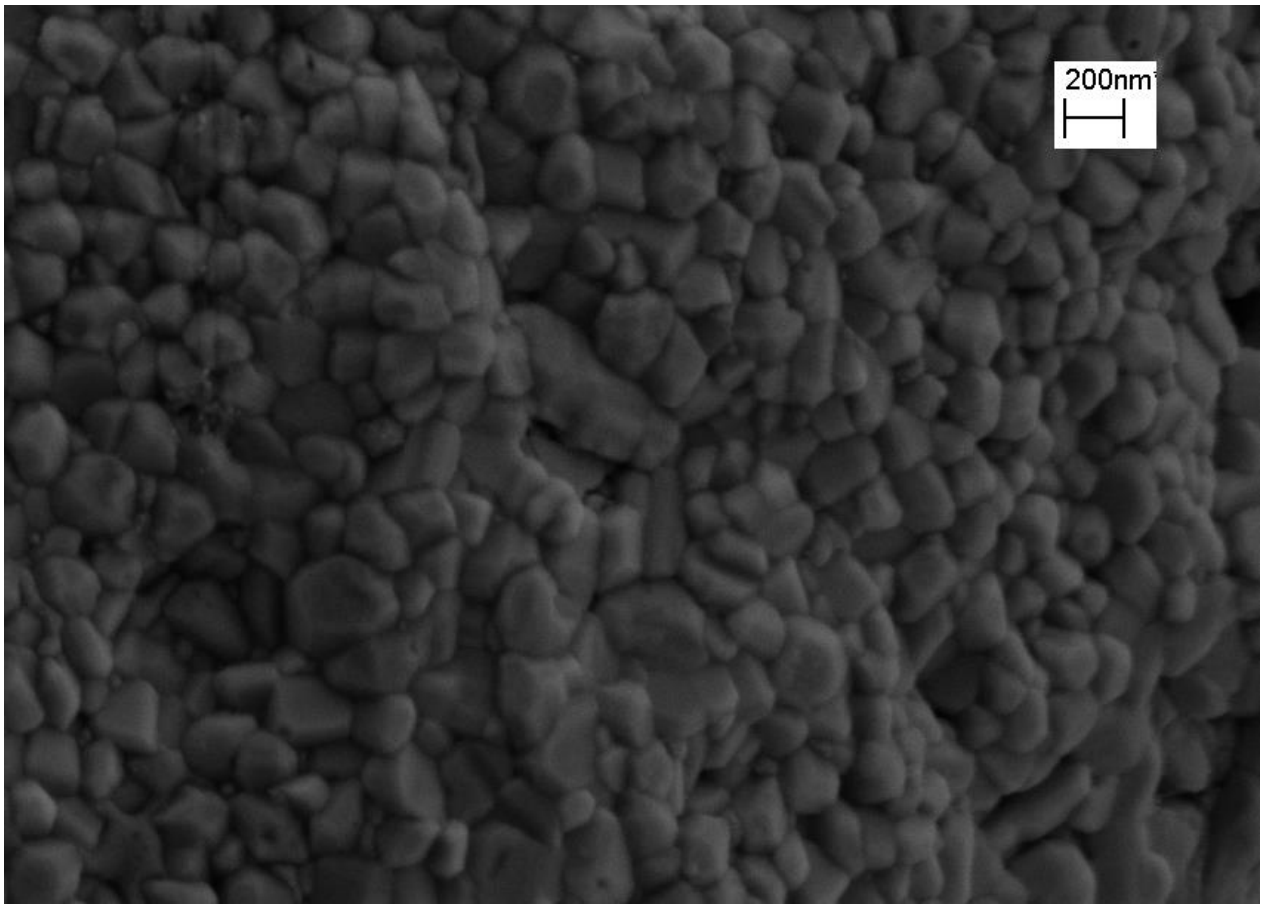


Figure 2-16 SEM imagery of nano crosslinked urease lyophilizate (nano CLEL), presenting the inferior morphology of nano particle units; 70.00 KX, EHT = 2.00 kV, WD = 8 mm, Secondary electron detector

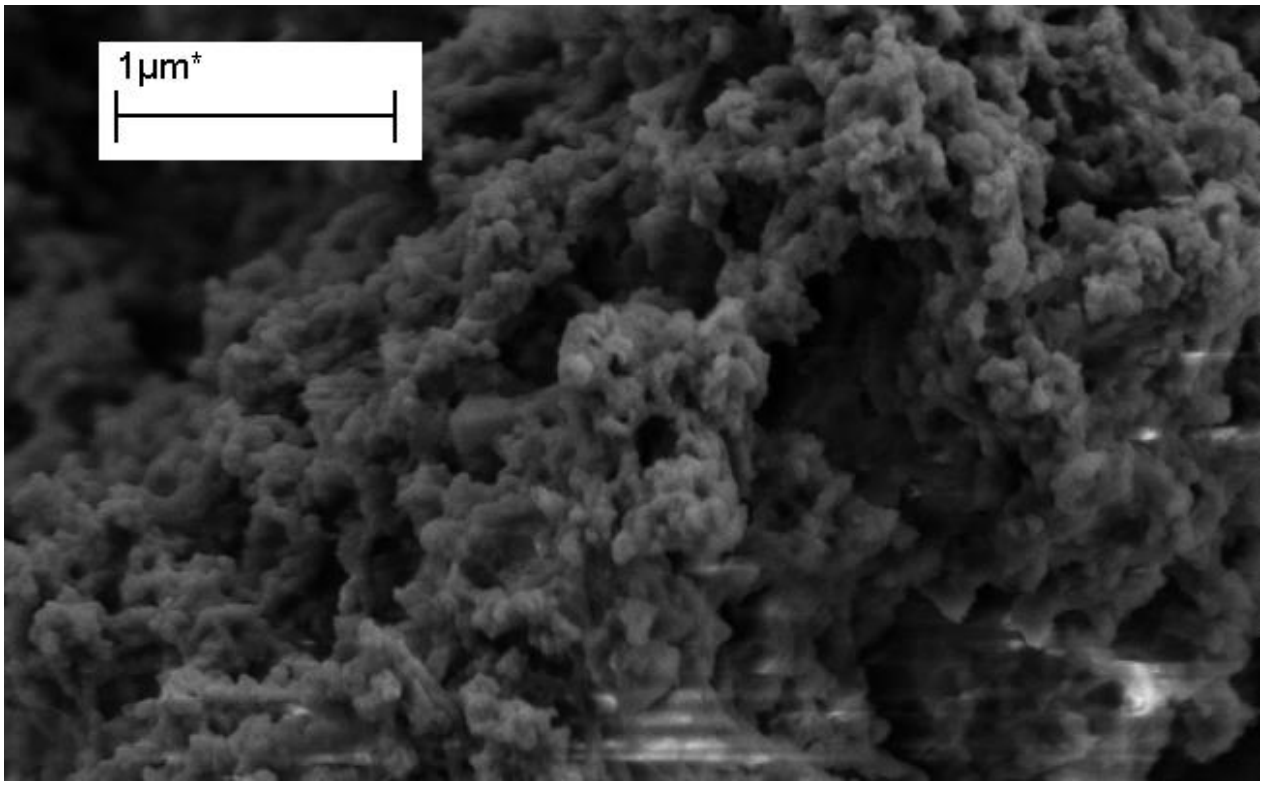


Figure 2-17 SEM imagery of nano crosslinked urease lyophilizate (nano CLEL), presenting the interior morphology of the nano particle unit; 50.00 KX, EHT = 2.00 kV, WD = 10 mm, Secondary electron detector (Sample E6.M1.C1)

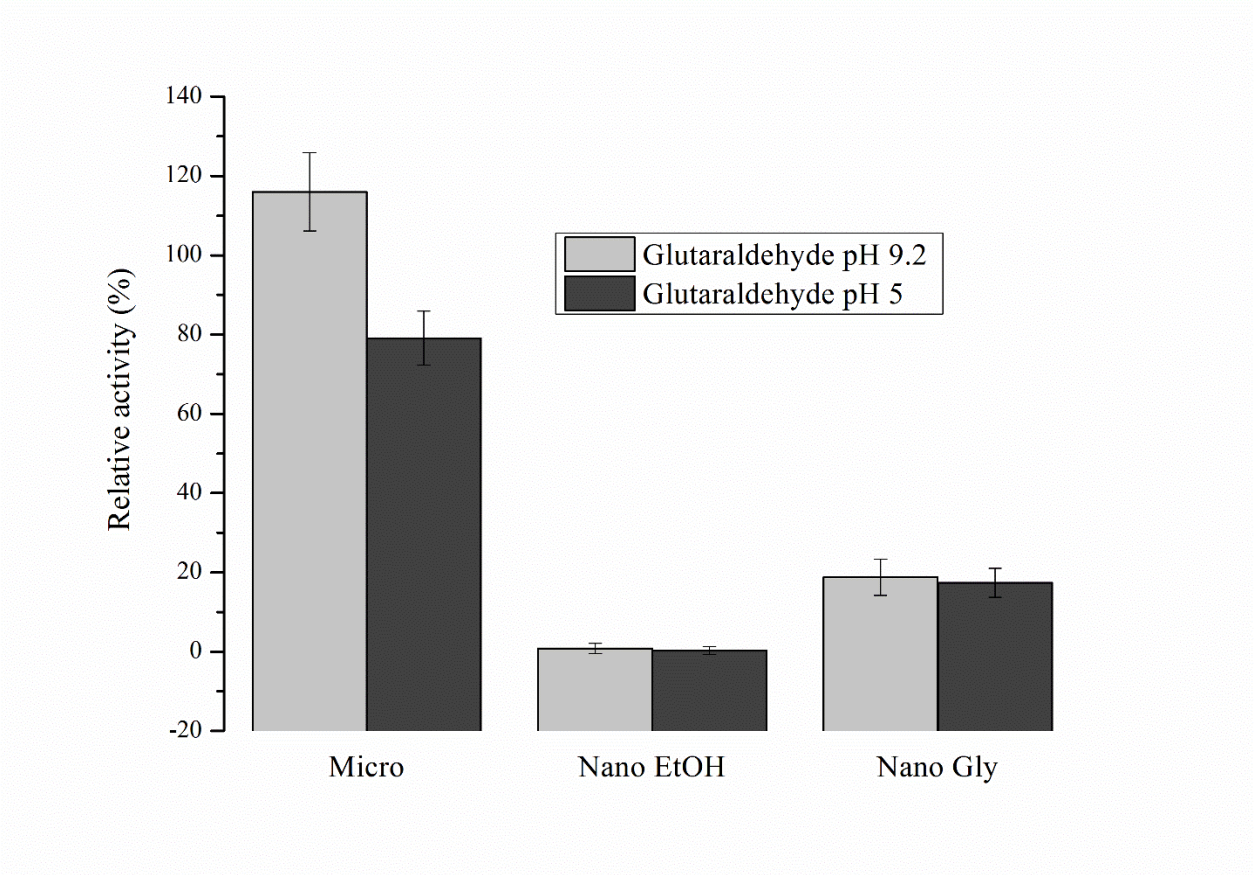


Figure 2-18 Catalytic activity comparison of urease CLEL and nano CLEL (1:3 urease to albumin weight ratio, crosslinking medium-saturated ammonium sulphate solution)

Nanonization procedure was performed in both 100% ethanol and 30% glycerol aqueous solution. As can be seen from Figure 2-18, nano CLEL products homogenized in 100% ethanol and 30% glycerol aqueous solutions show a deteriorated relative catalytic activity. Nano CLEA/CLEL catalysts are expected to enhance catalytic activities (%), by reducing the diffusion limitations [16] but it can be clearly observed that for urease CLEL samples, homogenization process in 100% ethanol and 30% glycerol aqueous solutions resulted diversely.

In the previous study (Zakharyuta, A., PhD Thesis, Nanosized Crosslinked Protein Aggregates (nano-CLPA)) it was shown that lyophilizate based formulations resulted in “softer” crosslinked end products, due to the higher porosity of the lyophilizate network as compared to that of an aggregate. Therefore, utilization of aqueous glycerol solutions as homogenization medium was

replaced by absolute ethanol. The first medium induced behaviour which could not be tolerated by these softer materials and resulted in irreversible conformational deformations, the second provided the "dry" environment resulting in brittle fracture of forming nano-particles and avoiding conformational deformation within the network under shear conditions. The described results were obtained on much more crosslinking friendly proteins, and it is safe to assume that the failure to retain catalytic activity upon nanonization urease CLEL is related to significantly lower crosslinking degree achieved. Therefore, it is clear that the large portion of the material did not withstand conditions dictated by shear forces necessary to generate nano particles.

2.4.3 Organic Reactions of Urease CLPA

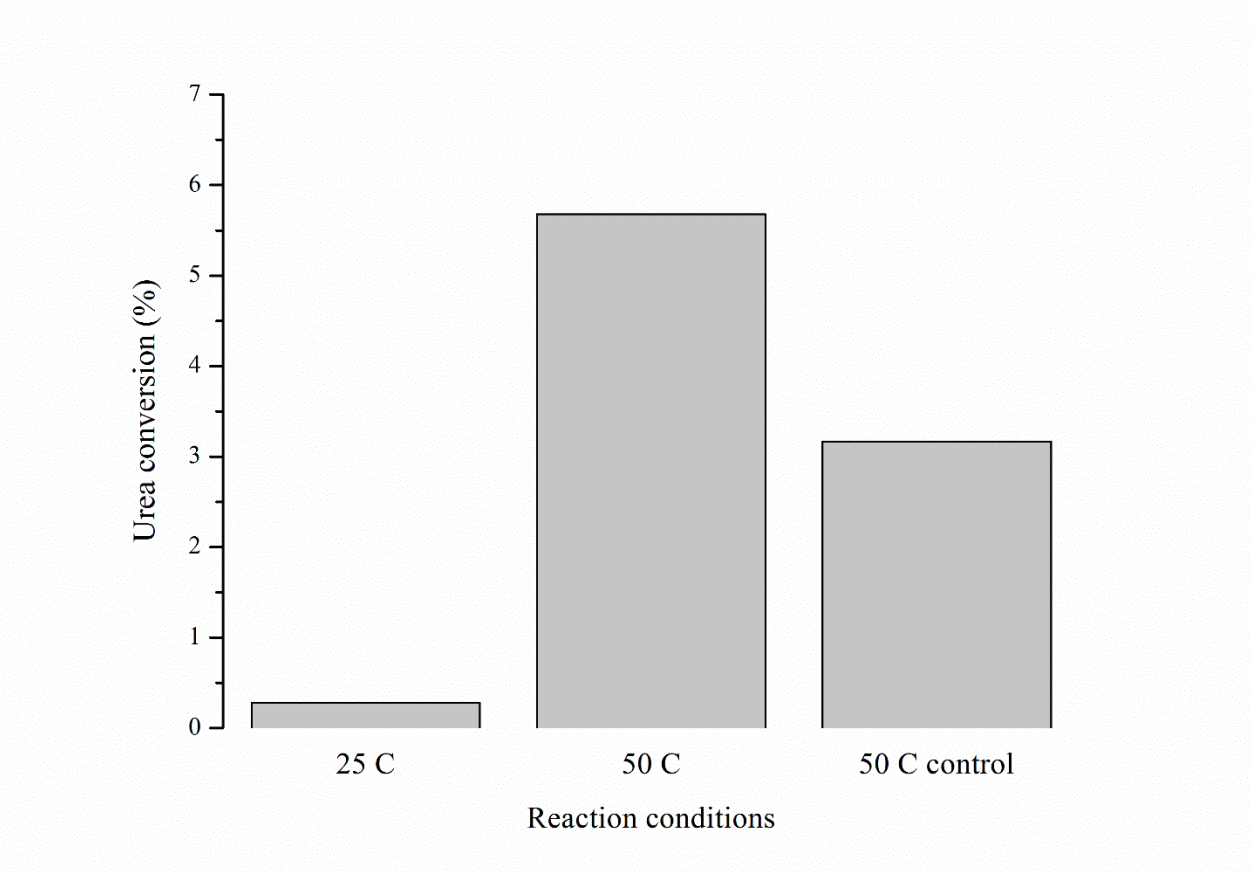


Figure 2-19 Urea conversion yield for dimethyl carbonate reaction

Figure 2-19 shows the urea conversion yields for dimethyl carbonate synthesis from urea and methanol with the reaction conditions 25 ^{o}C (with urease CLEL), 50 ^{o}C (with urease CLEL), and 50 ^{o}C control (without urease CLEL). As the boiling point of methanol is 60 ^{o}C, the highest reaction temperature was set as 50 ^{o}C. It is obviously seen that at higher temperatures, the urea conversion yield is higher with urease CLEL. Comparing the 50 ^{o}C urease CLEL and control samples, the urease CLEL is seen to double the yield of the control sample.

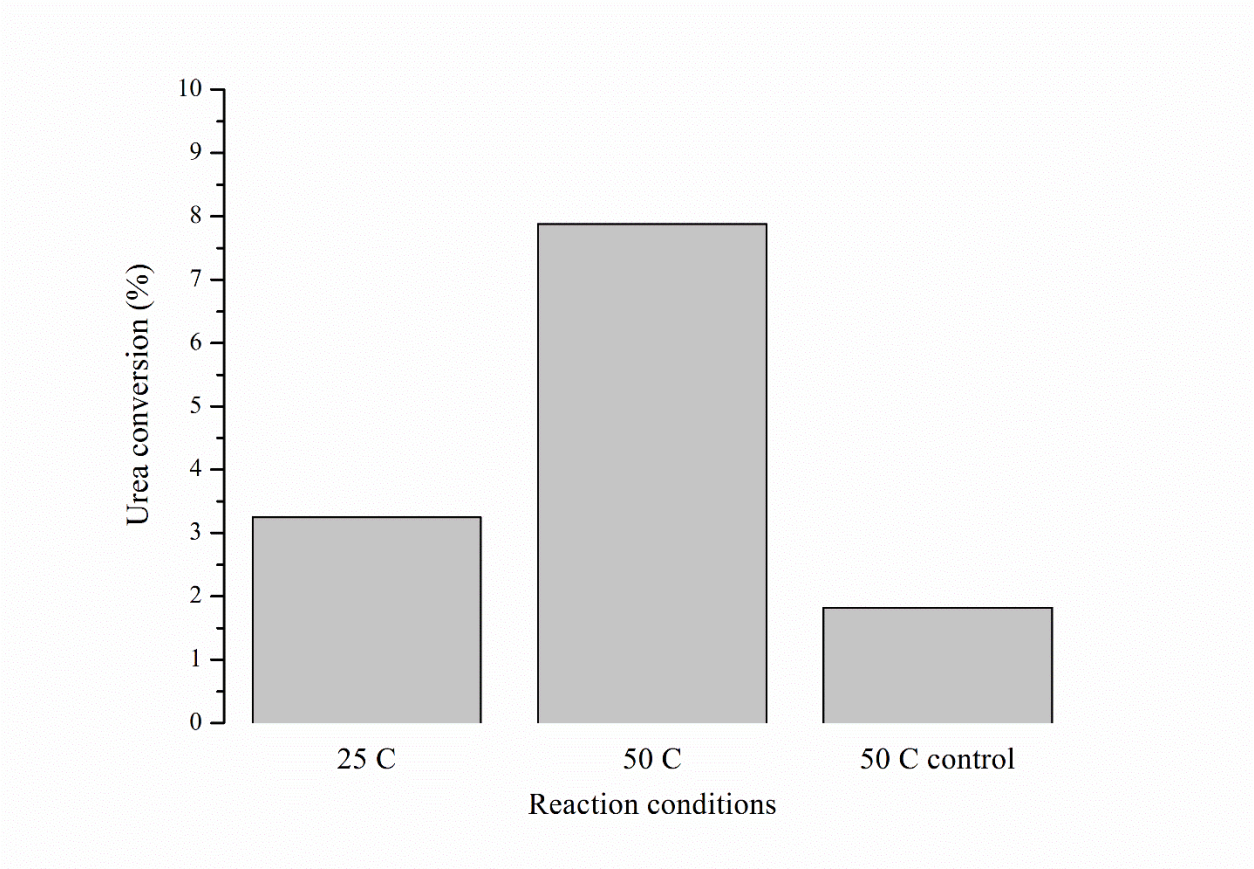


Figure 2-20 Urea conversion yield for ethylene carbonate reaction

Figure 2-20 shows the urea conversion yields for ethylene carbonate synthesis from urea and ethylene glycol with the reaction conditions 25 ^{o}C (with urease CLEL), 50 ^{o}C (with urease CLEL), and 50 ^{o}C control (without urease). It is obviously seen that at higher temperatures, the

urea conversion yield is higher with urease CLEL. Comparing the 50 ^{o}C urease CLEL and control samples, the urease CLEL is seen to be four times higher than the yield of the control sample.

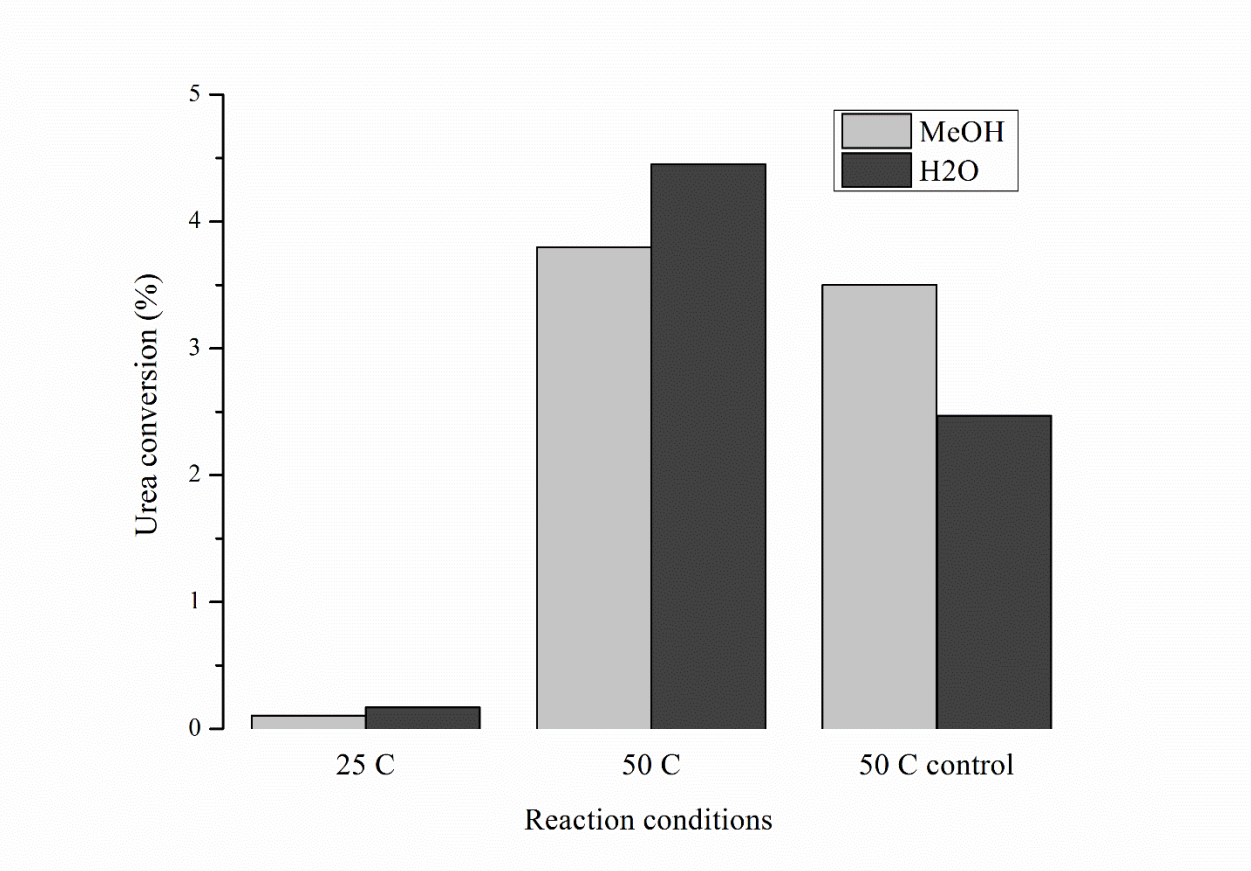


Figure 2-21 Urea conversion yield for carbodihydrazide reactions

Figure 2-21 shows the urea conversion yields for carbodihydrazide synthesis from urea and hydrazine performed in methanol and H_2O with the reaction conditions 25 ^{o}C (with urease CLEL), 50 ^{o}C (with urease CLEL), and 50 ^{o}C control (without urease CLEL). As the boiling point of methanol is 60 ^{o}C, the highest reaction temperature was set as 50 ^{o}C. It is obviously seen that at higher temperatures, the urea conversion yield is higher with urease CLEL.

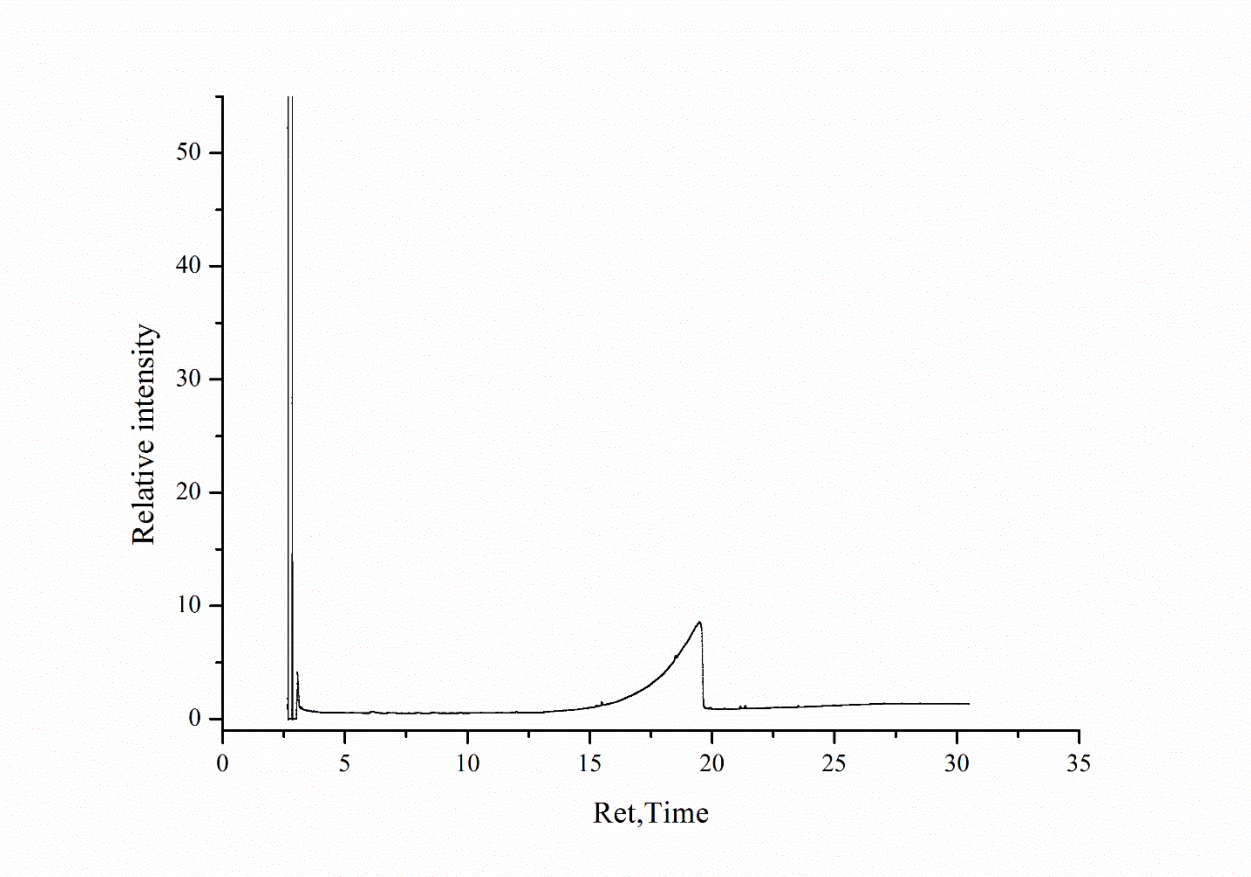


Figure 2-22 Gas chromatography for dimethyl carbonate product (2-30 min)

In figure 2-22, the gas chromotogram of the dimethyl carbonate product from the reaction of methanol and urea is shown. The broad peak around 15-20 minutes belongs to urea which has a high melting of 160 ºC. Dimethyl carbonate has a 90 ºC and methanol has a 60 ºC of boiling points. They are both observed at the peaks around 2-3 minutes.

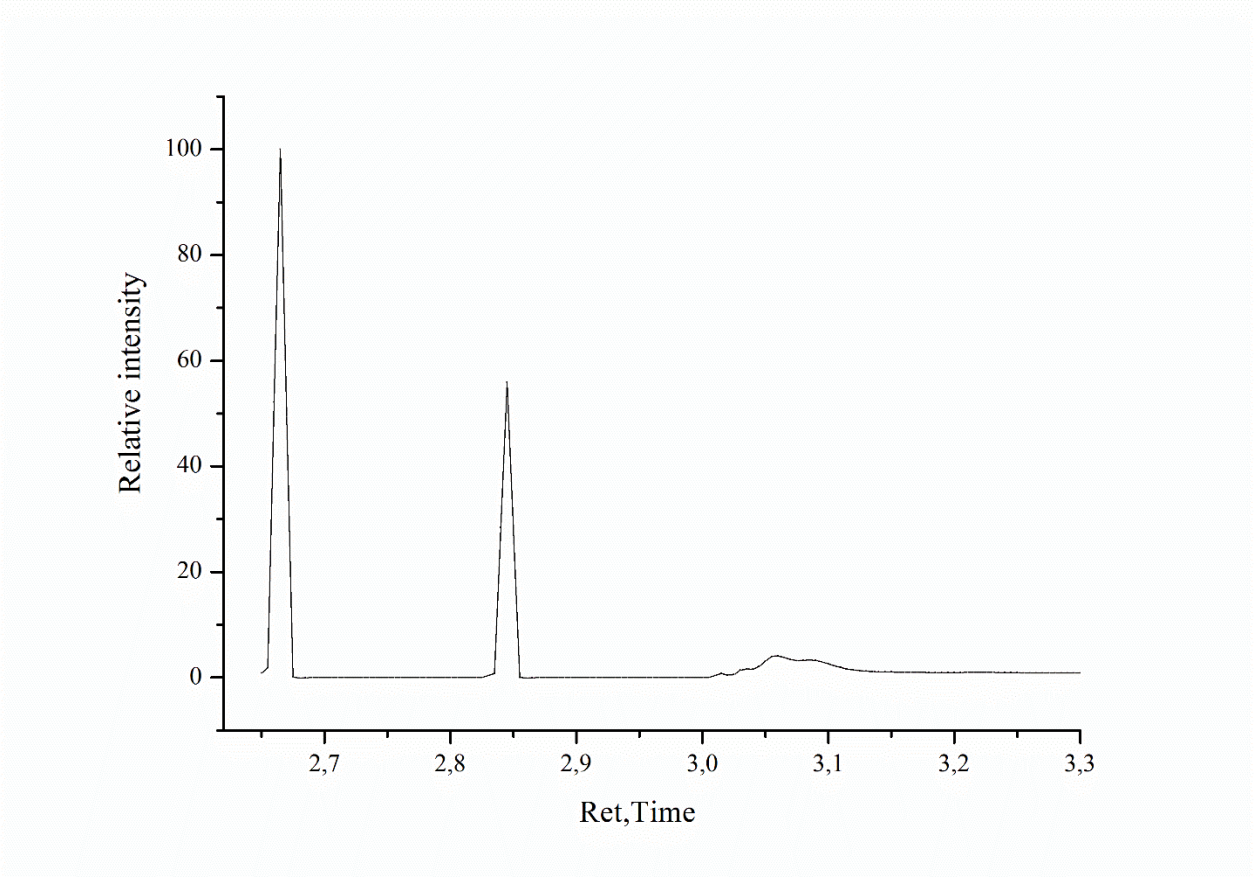


Figure 2-23 Gas chromatography for dimethyl carbonate product (2.5-3.3 min)

Zooming on the peaks around 2-3 minutes, shown on Figure 2-23, it was observed that the sharp peaks at 2.65 and 2.85 minutes belongs to the solvent, methanol, which contains isopropanol as an impurity of 1%. Dimethyl carbonate was hardly seen at minute 3.085 peak after several programs due to the fact that the solvent shades the peak of the final product.

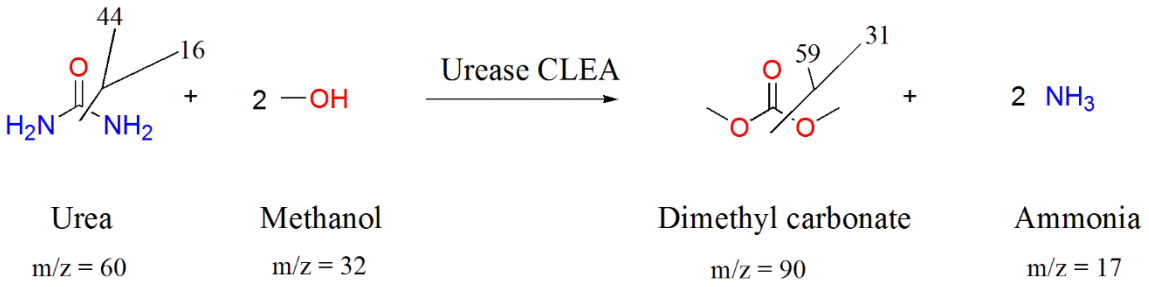


Figure 2-24 Fragment details for mass spectrum

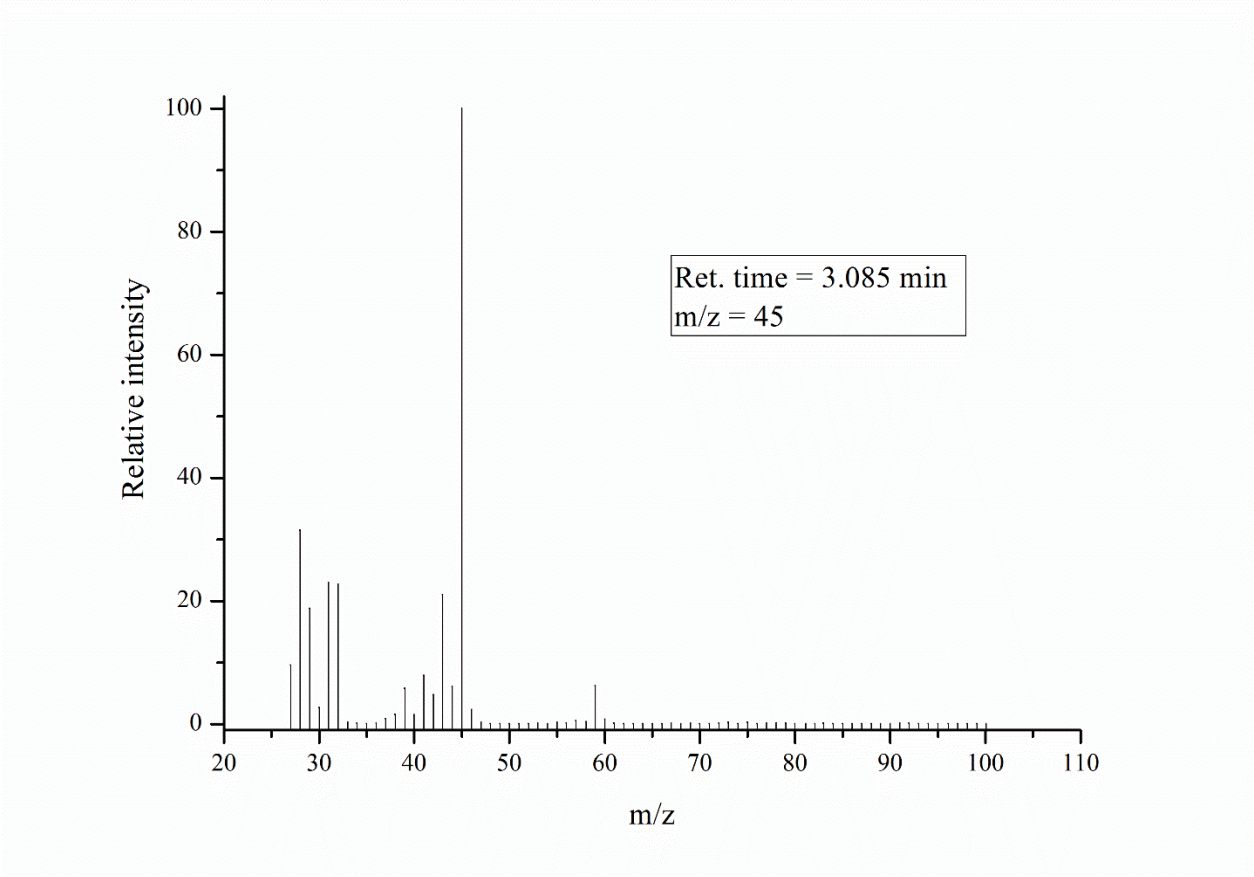


Figure 2-25 Mass spectrum for the peak retention time 3.085

Figure 2-25 shows the mass spectrum of the dimethyl carbonate. Checking the fragment details of the reagents from figure 2-24, it is quite obvious that we need to see the 59 and 31 peaks as a

proof of dimethyl carbonate. Because of the fact that the highest peak in the mass spectrum is 45, we can conclude that there is still urea in the reaction medium and it is not fully converted into product. The relative intensities (%) of the dimethyl carbonate peaks 59 and 31 are respectively 7% and 22% while the relative intensity (%) of the 45 peak, belonging to urea, is 100%. Although the urea conversion is not very high for this reaction, this data can be integrated with the data coming from Figure 2-18. At 25 ^{o}C, the urea conversion yield is not very high compared to the yield of the reaction at 50 ^{o}C.

2.5 Concluding Remarks

In this chapter, conventional urease CLEA methods were described. These methods led to dramatically low aggregation and cross linking yields, and displayed statistically insignificant catalytic activity of the immobilized urease product. The reason for poor precipitability of urease solutions remains unclear. The greatly declined catalytic activity of recovered CLEA, are explained by failure to stabilize the protein within the aggregation procedure prior to crosslinking. Furthermore, the poor crosslinking outcome appeared to be related to an unusual structure, which discouraged surface functional group interactions with crosslinker. The situation was markedly improved by the partial crosslinking of urease in the solution phase prior to aggregation and heterogeneous phase crosslinking. More importantly, however, a critical breakthrough was achieved by through substitution of aggregate precursors by lyophylizates and subsequent cross linking in an antisolvent or salt, providing near complete protein recovery. Moreover, the subsequent crosslinking of the lyophilizate (yielding a CLEL) in a suitable antisolvent bearing the crosslinker led to a much improved crosslinking yield and catalytic activity.

Applicability of urease CLEA in synthesis catalyst in an organic solvent has been shown. Improvement of catalysis rate remains the subject for further optimization in the future work.

CHAPTER 3 Pepsin Cross Linked Enzyme Aggregates (CLEA) and Nano Cross Linked Enzyme Aggregates (nano CLEA)

3.1 Introduction

Pepsin, acidic protease, is one of the most widely used industrial proteases[39]. It has been routinely used in food, pharmaceutical, leather, cosmetic and textile industries[40]. However, some limitations exist in application spectra of pepsin enzyme in industry in terms of pH stability, thermal stability and etc. These limitations lead to need for improvements of enzymatic activity of pepsin with several engineering methods as mentioned in Chapter I. In here, we produced the first cross-linked pepsin nanoaggregates in order to alter the enzymatic properties of this catalyst for further applications.

Firstly, the active site of pepsin has been well described by Sielecik et al. upon refinement of its crystal structure. It has been indicated that Asp32 and Asp215 residues are located on hydrophobic core of pepsin and acted as catalytic residues. It has been proposed that the hydrogen networking has been formed around active site residues such as Asp32-Ser35 with 2.8 Å and Asp215-Thr218 with 3.4 Å [41]. In active site cleft of pepsin, there are found solvent sites[41]. Sielecki et al. described the activation mechanism of pepsin by the fact that the nucleophilic attack on the carbonyl carbon atom of substrate have been performed by catalytic residues and this catalytic pathway requires the presence of water in active cleft where the enzyme-substrate complex bound[41]. In active cleft of pepsin, there were found two specific water molecules and these water molecules are interacted with Glu107 and Ser104, located in hydrophobic cavity of pepsin. In here, Glu 107 is pointed toward the hydrophobic cavity and contributed to strong hydrogen networking. Thus, this contribution results in protonation of Glu107, acted as proton donor to another water molecule at hydrophobic cleft in further step.

Moreover, the importance of salt concentration of environment on catalytic activity of pepsin was reported. It has been revealed that high salt concentrations disrupt the conformation of active site cleft of pepsin and lead to great reduction in its enzymatic activity. However, as the optimum pH environment was provided to pepsin enzyme, the favourable interaction between Glu287 and

substrate was formed upon the protonation of this residue. As similar to other aspartyl proteases, the active site cleft of pepsin is long enough to coordinate substrate through seven or eight residues. Sielecki et al. also described the substrate binding mechanism of pepsin and revealed that there were found two large flats in pdb structure of pepsin[41]. In the first flap, Tyr75 and Thr77 residues interact with substrate and there are found Val291 and Leu298 residues in the second flap, which are directly interacted with amino portion of substrate[41].

While no significant challenge was posed by pepsin in terms of aggregation, the crosslinking step is highly challenged. The obvious explanation can be derived by observing the structure, namely appearance of a single Lys residue available for conjugation.

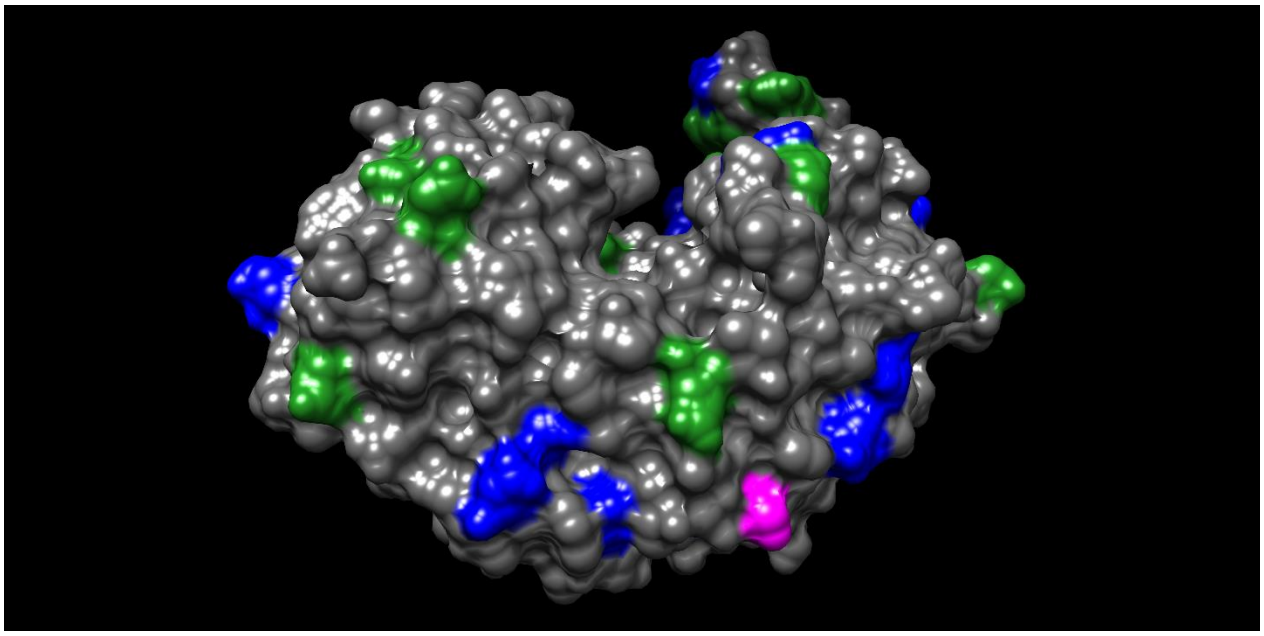


Figure 3-1 pdb structure of pepsin (pdb code: 5pep); Lys: Magenta, Asp: Blue, Glu: Green [41]

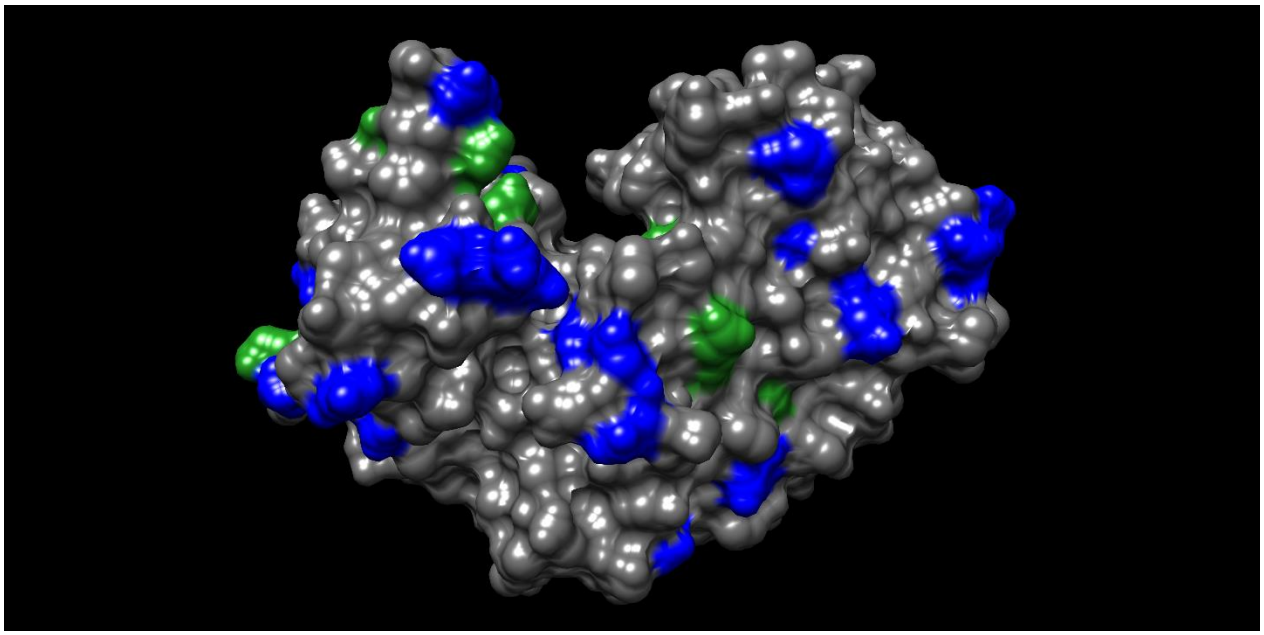


Figure 3-2 Presentation of the active site of pepsin (pdb structure) (pdb code: 5pep); Asp: Blue, Glu: Green [41]

3.2 Materials

Instrumentation:

Thermo Scientific Nanodrop 2000 Spectrophotometer

The rest of the instrumentation can be seen in Chapter 2.2 Materials section.

Chemicals:

Pepsin (EC 3.4.23.1), from porcine gastric mucosa was obtained from Sigma-Aldrich.

Bovine Hemoglobin was obtained from Sigma-Aldrich. Hemoglobin from bovine blood, suitable for protease substrate, substrate powder.

Trichloroacetic acid solution 6.1 N was obtained from Sigma-Aldrich.

Dextran (native) was obtained from abcr GmbH&Co.

The rest of the chemical reagents can be seen in Chapter 2.2 Materials section.

3.3 Methods

3.3.1 Pepsin CLEA Synthesis

Starting solution was prepared with pepsin powder (50 mg/ml) and albumin (0-50 mg/ml) in 10 mM $CaCl_2$/10 mM HCl pH4 buffer (4 ^oC).

Herein, both the lyophilization and the aggregation methods were attempted.

3.3.1.1 Pepsin CLEL Synthesis via Lyophilization Method

For the lyophilization method, the solution was transferred into 2mL capacity Eppendorf tubes, and the tube rack was submerged in liquid nitrogen followed by lyophilization (24h). After the lyophilization procedure, the lyophilizates were dropped into crosslinker-precipitant mixture (25% glutaraldehyde pH 5 / 12.5% glutaraldehyde pH 9.5 / dextran polyaldehyde / N,N'-dicycylohexylcarbodiimide – acetone / isopropanol / 4M ammonium sulphate). The reactions took place under 4 ^oC / -20 ^oC (20 h, 500 rpm). Following the centrifugation (5 min, 10 krpm), the pellet was treated with a freshly made aqueous solution of sodium borohydride (1000 μl, 1mg/ml dH_2O, 30 min), centrifuged (10 krpm, 5 min), and twice reconstituted (1000 μl, RT, 5 min) and centrifuged (10 krpm, 5 min) in distilled water to remove traces of reagent. The wet pellet was dried under vacuum (RT, 12 h).

Crosslinker reagents, aggregation mediums and protein composition variants for pepsin CLEL products are summarized in the table below:

Table 3-1 Enzyme concentration, crosslinker and aggregation medium information for pepsin CLEL formation via lyophilization method

Enzyme solution code	**P1**	**P2**		
Concentration /ml	50 mg pepsin	50 mg pepsin 50 mg albumin		
Crosslinking medium code	**M1**	**M2**	**M3**	
Crosslinking medium	4M ammonium sulfate	Isopropanol	Acetone	
Crosslinker code	**C1**	**C2**	**C3**	**C4**
Crosslinker	Glutaraldehyde 25% pH 5	Glutaraldehyde 12.5% pH 9.2	Dextran polyaldehyde	N,N'-dicyclohexylcarbodiimide

3.3.1.2 Pepsin CLEA Synthesis via Aggregation Method

For the aggregation method, protein solutions were dropwise added into a saturated ammonium sulfate solution (salting out) or an anti-solvent (isopropanol, acetone) under constant stirring conditions at v:v ratio of 1:9 or 1:5 and left to stir for 20-30 min at 400-550 rpm, at 4 ºC. Following this procedure, the crosslinker, 12.5 wt% pH 9.2 glutaraldehyde, N,N'-dicyclohexylcarbodiimide, dextran polyaldehyde was dropped into enzyme aggregate crosslinking reaction step was subsequently permitted for 20 h (4 ^{o}C / -20 ^{o}C). The biological material was recovered as a pellet following centrifugation (5 min, 10 krpm), the pellet was treated with a freshly made aqueous solution of sodium borohydride (1000 µl, 1mg/ml dH_2O, 30 min), centrifuged (10 krpm, 5 min), and twice reconstituted (1000 µl, RT, 5 min) and centrifuged

(10 krpm, 5 min) in distilled water to remove traces of reagent. The wet pellet was dried under vacuum (RT, 12 h).

Crosslinker reagents, aggregation mediums and protein composition variants for pepsin CLEA products are summarized in the table below:

Table 3-2 Enzyme concentration, crosslinker and aggregation medium information for pepsin CLEA formation via aggregation method

Enzyme solution code	**P1**	**P2**		
Concentration /ml	50 mg pepsin	50 mg pepsin 50 mg albumin		
Crosslinking medium code	**M1**	**M2**	**M3**	
Crosslinking medium	Acetone	Isopropanol		
Crosslinker code	**C1**	**C2**	**C3**	**C4**
Crosslinker	Glutaraldehyde 12.5% pH 9.2	N,N'-dicyclohexylcarbodiimide	Dextran polyaldehyde	

Crosslinkers

Glutaraldehyde

Glutaraldehyde was applied at concentrations of 2.12 x 10^{-5} mol per mg protein (dry weight equivalent). Glutaraldehyde is typically stored and sold at slightly acidic pH values, which serves to reduce its optimal reactivity. In this work, commercial glutaraldehyde stocks (25wt%, pH 5) were directly used without pH adjustment. Alternatively, water-diluted stocks (12.5wt%, adjusted

to pH 9.2 using 0.1M sodium carbonate buffer and pH 7.4 using 0.1M sodium phosphate buffer) were used.

Dextran polyaldehyde (PDA)

Dextran polyaldehyde was synthesized in house according to the following procedure:

Dextran 1.65 g was dissolved in 80 mL of water, and 3.85 g sodium metaperiodate were added. The resulting solution was stirred at room temperature during 90 min. Subsequently, the solution was dialyzed five times, using a MW cutoff of 10 KDa against 5 L of water each time at room temperature during 2 hrs and under stirring. The final volume of the dextran polyaldehyde was 87 mL.

Dextran polyaldehyde was applied at concentration of 0.76 mg per mg protein (dry weight equivalent) for all samples, if not stated otherwise in the text.

N,N'-dicyclohexylcarbodiimide

N,N'-dicyclohexylcarbodiimide (DCC) was applied at 1.9×10^{-5} mol concentrations per mg protein (dry weight equivalent), with prior addition of N-hydroxysuccinimide (2.8×10^{-5} mol).

3.3.2 Nano Pepsin CLPA Generation

Nanonization was achieved via hydrodynamic shear application using homogenizer (Heidolph silent crusher M) with varying processing time and shear conditions. In a typical run, CLEA (2.5 mg) was dispersed in 1 ml 30% aqueous glycerol solution or 100% ethanol and nanonization was performed using different instrumental settings and times (10-21 krpm; 30-60 min). Given the thermal stability of CLEAs, no elaborate steps were taken to avoid incidental heating of the dispersion during nanonization.

The nano-CLPA samples were transferred into 1.5 ml eppendorf tubes with the dialysis membrane replacing the top of the tube cap, tubes were further secured with parafilm tape to avoid any leakage. All samples prepared as described were dialyzed against 10 mM $CaCl_2$ 10 mM HCl pH4 buffer, with constant agitation, for the period of 6 hours, repeated 4 times. (Snake

Skin® Dialysis Tubing, 3.5K MWCO, 35 mm dry I.D, 35 feet was obtained from Thermo Scientific).

3.3.3 Characterization of Pepsin CLPA and Nano Pepsin CLPA

The instrumental analysis was performed via Dynamic Light Scattering and Scanning Electron Microscopy.

3.3.3.3 Dynamic Light Scattering Measurements

The sample was diluted 10 fold in medium corresponding to their homogenization conditions. DLS data was collected on samples equilibrated at 25 ºC in 2ml disposable cuvettes, as a result of 3 consecutive scans, Malvern Zetasizer NANO ZS. Absorption of each sample was measured at 633 nm and included in DLS measurement protocol. Particle refractive index of 1.5 was assumed for all CLPA samples and refractive index of corresponding medium was included in the protocol. Data was analyzed using protein analysis model, Malvern Zetasizer software.

3.3.3.4 Scanning Electron Microscopy Analysis

CLPA powder was subjected to treatment in a Cressington Sputter Au/Pd Coater. An approximate coating thickness of 2-3nm was targeted. The processed samples were loaded into the vacuum chamber of a ZEISS brand LEO SUPRA 35VP model SEM with GEMINI column. An electron gun voltage of 2kV was employed throughout the analyses.

3.3.3.5 Pepsin Catalytic Activity Assay Protocol

For the determination of the pepsin activity, modified Worthington assay based on the stop-point assay of hemoglobin degradation developed by Anson (1938) was applied to the samples [42].

1 ml of 1 mg/ml CLEA and 1 ml of 1 mg/ml native pepsin solutions in 0.01 N HCl were dropped into 5 ml of 2.5 w/v hemoglobin (4:1 diluted with 0.3 N HCl). After 20 minutes of incubation at 37 ºC, 10 ml of trichloro acetic acid (TCA) was added to the solutions. After 5 minutes of incubation, samples were spinned for 5 minutes at 13 krpm. The absorbance values of the supernatants were measured at Thermo Scientific Nanodrop 2000 Spectrophotometer. Relative

activity (%) was compared by assessing the pepsin mass fraction within each CLEA against an equal mass of freely soluble native pepsin. The native pepsin was arbitrarily assigned a value of 100%.

3.4 Results and Discussion

In this chapter, challenges associated with crosslinking step of cross-linked pepsin aggregates were addressed.

Both CLEA and CLEL formulations have been considered, but as opposed to urease formulation no dramatic improvement was observed with incorporation of CLEL technique. On the other hand crosslinker choice and physical reaction conditions appeared much more influential.

3.4.1 Pepsin CLPA Synthesis

The crash precipitation efficiencies of pepsin appeared highly dependent on the medium, with most conventional saturated ammonium sulphate precipitation leading to intolerable protein losses. The most efficient antisolvents, in terms of precipitation yields were isopropanol and acetone, yielding an average of 80-90% of initial protein amount in the form of aggregate (results not shown). Subsequent cross linking step was successfully facilitated in isopropanol, while crosslinking in acetone led to dramatically declined yield. Significant decrease in catalytic activity of obtained CLEA was observed in case of both antisolvents, more dramatically pronounced with the use of acetone (Figure 3-2). Therefore for all further formulations isopropanol was utilized as the aggregation medium.

Furthermore co-precipitation of pepsin with albumin did not lead to significant improvement, in terms of aggregation and crosslinking efficiency. On the other hand, while statistically comparable, results obtained from co-precipitate CLEAs were generally exceeding those of pepsin aggregates. Therefore, data corresponding to co-precipitate samples is presented herein.

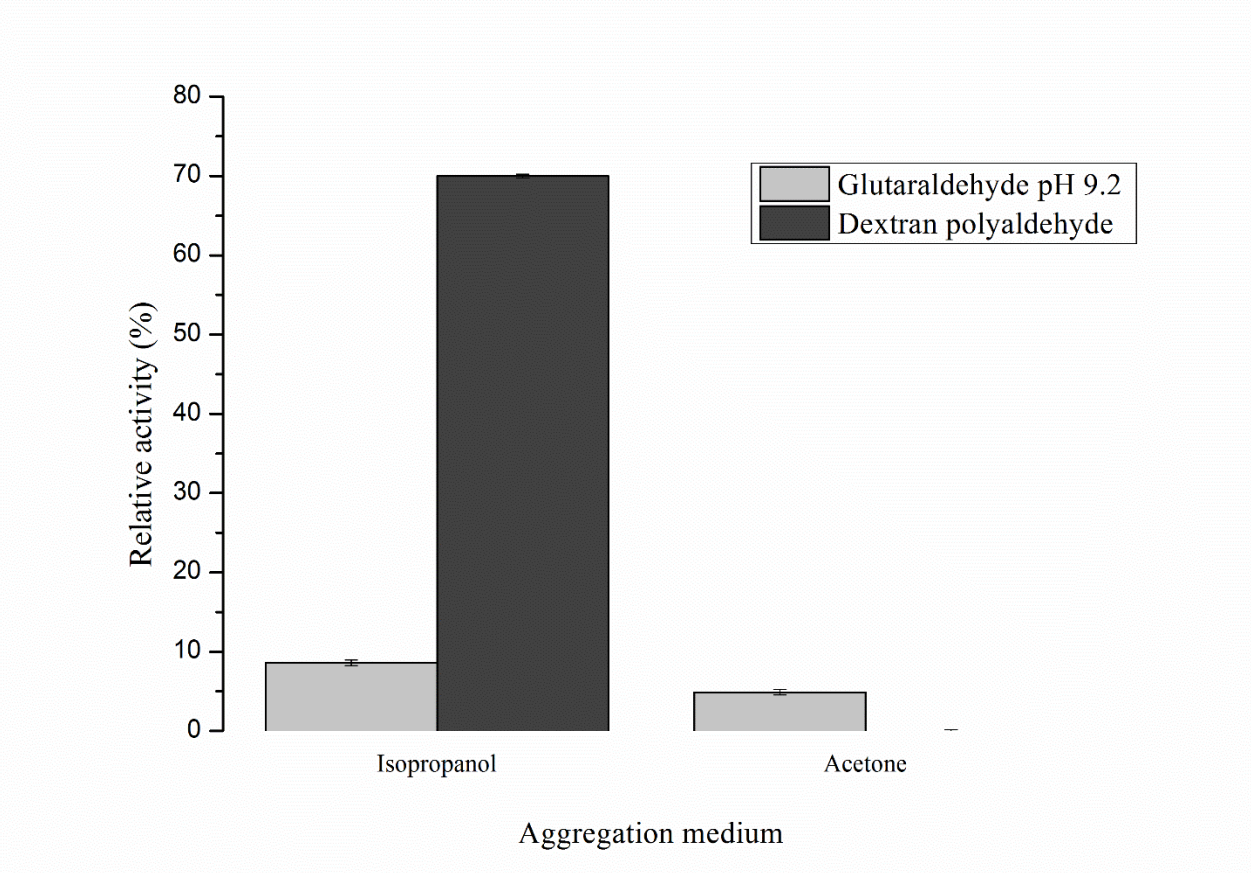


Figure 3-3 Effect of aggregation medium and glutaraldehyde reagent pH on relative catalytic activity of pepsin CLEA

Comparison of different crosslinker reagents efficiency has led to the conclusion that dextrane polyaldehyde was the most suitable reagent in terms of catalytic activity conservation (Figure 3-3), as retention of 70-80% catalytic activity could be achieved. Most conventional glutaraldehyde reagent at various reagent pH values all yielded 0-10% of native enzyme activity, with pH 9.2 providing the highest value. Utilization of carbodiimide reagent has led to slight improvement of activity retention as compared to glutaraldehyde. The most plausible results were obtained through incorporation of reaction intermediate stabilizing hydrosuccinimide reagent reaching the maximum of 30%, as described in methods section of this Chapter.

Furthermore, incorporation of subzero temperature conditions throughout the crosslinking procedure did not benefit glutaraldehyde and carbodiimide crosslinking modes. On the other hand, dextrane polyaldehyde crosslinking at -20 ^{o}C provided CLEA with somewhat increased catalytic activity as compared to products of reactions conducted at conventional 4 ^{o}C.

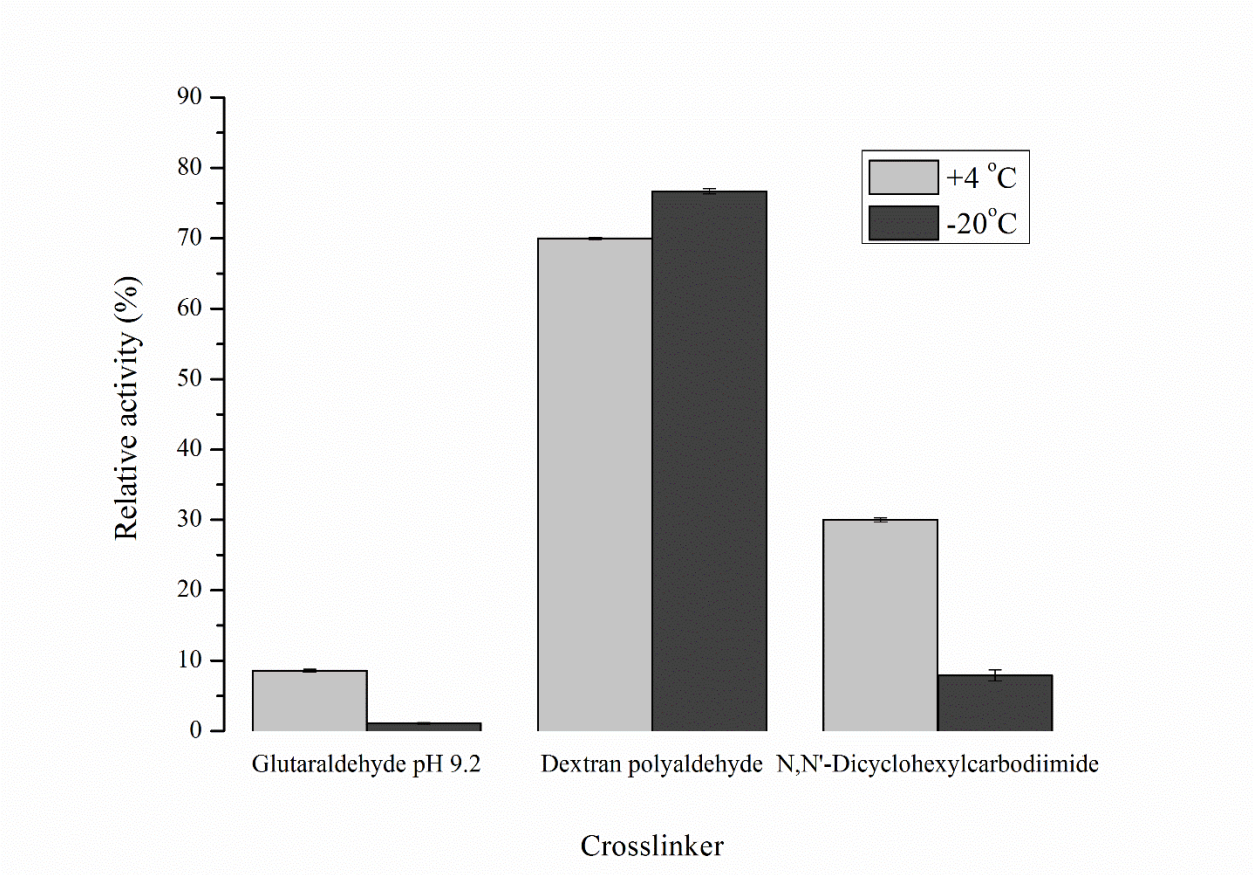


Figure 3-4 Effect of cross linking reagent and cross linking temperature on relative catalytic activitiy of pepsin CLEA. (Crash precipitation facilitated by isopropanol)

It followed to reason that a combination of the large bulk and low temperature could have permitted the close interaction of protein and crosslinker, leading to secondary benefits such as crosslinker interactions with surface hydroxyl groups, and mechanical fastening of the structures to one another by way of encapsulation and intertwining. Indeed, substitution of dextran polyaldehyde by the more established glutaraldehyde resulted in a much poorer outcome, attesting to the importance of steric bulk.

One of the main reasons behind improved enzymatic activity is that low temperature contributes to maintain a fixed superstructure of an enzyme, which can be further preserved upon cross-linking agents [15]. The highest level of structural preservation at low temperature makes possible that cross-linked agent reaches the fixed super-structure of an enzyme, which contributes to preservation of enzyme activity. Furthermore, much declined autolysis should be expected throughout the crosslinking period, leading to more efficient preservation of overall structural integrity of the pepsin aggregate bulk.

It has been well indicated in literature that there are some limitations in cross-linking of enzyme in an effective ways such that the limiting number of binding sites or steric inaccessibility in enzyme structures [43]. As shown in Figure 3-4, there is only one lysyl residue on surface of the protein, which is essential for cross-linking reaction.

As mentioned before, the enzyme precipitation is one of the integral parts of cross-linking procedures. Up to now, many different ways have been reported to precipitate enzymes effectively such as cooling, pH adjustments, addition of organic solvents, immunoprecipitants and etc. Among these factors, cooling in organic solvents is one of the most effective methods for protein precipitation since the synergistic effects of low temperature and organic solvent are well observed. In organic solvents, some diffusional limitations are observed due to excess rigidity of enzymes [44] but the high stability of enzymes are mostly reported upon improvements of intra-molecular forces in enzyme. When the diffusional limitation and low solubility of enzymes due to organic solvent and low temperature, respectively, were well combined, the synergistic effects are clearly observed and report to improvements in cross-linked enzyme activity. To overcome the rigidity problem of enzymes in organic solvent, as well known in literature, organic solvent imposes some degree of rigidity on enzyme structure. In order to solve this problem effectively, some practical solutions are reported such as addition of small amount of water to reaction environment. It has been already reported in literature that the addition of small amount of water to reaction environment provides enough flexibility for proper enzyme functioning without altering chemical reaction type [45]. Thus, this became the motivation to perform cross-linking reaction in 95-5 % (v/v) organic solvent: water environment to provide enough structural flexibility to the enzyme also for further proper functioning. The cross-linking results performed

at -20 ^{o}C in 95/5 % tells us that the addition of 5% water to reaction environment works well in terms of providing required flexibility to enzyme, especially Lys residues available on surface and we get effectively cross-linked enzyme aggregates. The reason behind improved CLEA activity in 95/5 % (v/v) reaction environment can be explained by the fact that the addition of small amount of water, as 5%, leads to lesser rigidity on surface of (pepsin/urease) compared with that of in 100% organic solvent. In addition to surface rigidity, addition of 5% water to reaction environment might Thus, Lys residues located on surface, crucial and essential for cross-linking, have had a high degree of freedom for any possible chemical attack in 95-5% (v/v) organic solvent-water environment, compare with that of in 100% organic solvent environment. Moreover, addition of 5% water to reaction environment contributes to preservation of catalytic site as if it is in water environment. Compared to 100% organic solvent environment, it is expected to observe less exposition of catalytic triad toward solvent in 95/5 % (v/v), compared with 100% organic solvent. Thus, the high degree of catalytic triad preservation is well combined with higher degree of flexibility of Lys (surface) with 95/5 % system. Providing higher degree of flexibility to Lys residues on surface contributes well to cross-linking efficiency of enzyme.

Keeping with the argument above, ironically, anti-solvent induced rigidity and neighbor- and crosslinker-induced restrictions of conformational freedom will also reduce the protein's entropy per unit time. The end result would be Gibbs ground state elevation of individual proteins in the CLEA, yielding more reactive catalysts. This statement would support the enhanced overall rigidity effect in favor of CLEA catalytic activity. Furthermore, incorporation of aqueous fraction into crosslinking medium could potentially contribute to some enhancement of earlier mentioned autolysis of the protease, through introduced flexibility.

While the weight-averaged contributions of these terms are subject to variability, it would appear, given the net rise of activity in dextran polyaldehyde facilitated CLEAs in case of both 4˚C 100% anti-solvent induced aggregates and 95% anti-solvent crosslinked at -20˚C, that catalytic losses due to rigidity of an aggregate precursor had not substantially contributed. Nevertheless, the discussion above provides further opportunities to optimization of the procedure, aiming full retention or even relative increase in pepsin CLEAs.

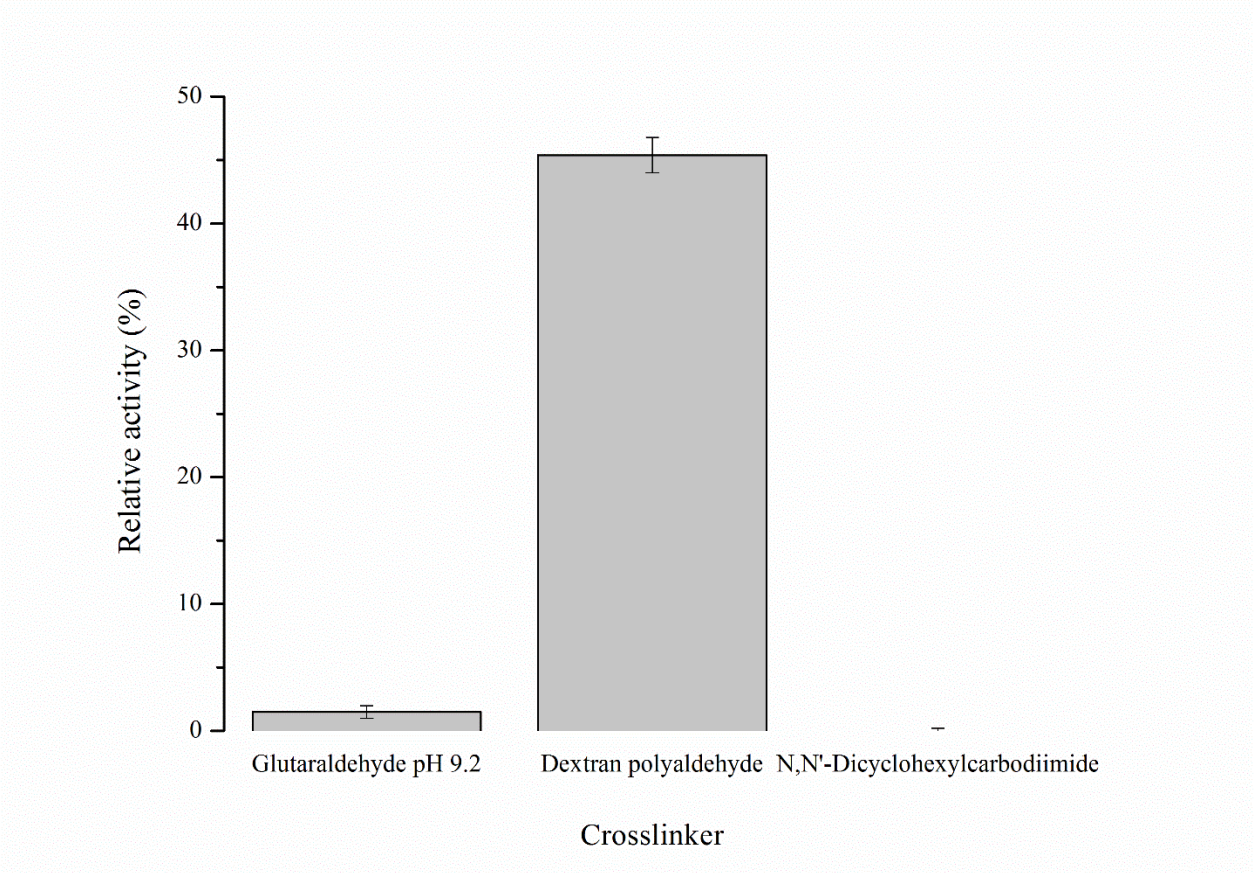


Figure 3-5 Effect of cross linking reagent on relative catalytic activitiy of pepsin CLEL. (Crosslinking medium-isopropanol, 4 ^{o}C)

As can be observed from Figure 3-5, method of lyophylizate crosslinking (yielding CLEL) earlier rather successfully demonstrated on the example of urease, has not proved beneficial in case of pepsin formulations. The obvious advantage of dextrane polyaldehyde utilization is once again well pronounced in resultant CLEL formulations. It could be rationalized, that the rigidity of an aggregate system formulation as opposed to, porosity related, relative flexibility of lyophilizates, introduces the necessary thermodynamic stress contributing to improvement of catalytic activity. Furthermore more closely packed structure of aggregates facilitated the necessary proximity of available juxtaposed crosslinkable groups, therefore maximizing the overall crosslinking degree.

3.4.2 Nano Pepsin CLPA Synthesis

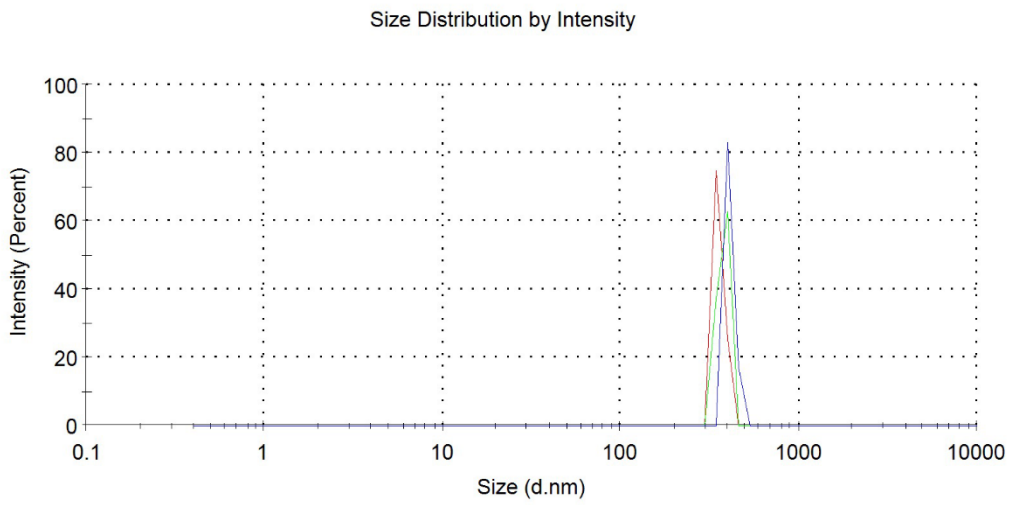


Figure 3-6 DLS result of nano crosslinked pepsin aggregate homogenized in absolute ethanol, at 21krpm for 30 min (nano CLPA) (Albumin:Pepsin 1:1 (w:w) Acetone, glutaraldehyde pH 9.2 case)

Figure 3-6 presents a DLS measurement of supernatant (1krpm) of a urease CLEA suspended and homogenized in 100% ethanol. This data provided evidence of nanoparticle content generation upon nanonization procedure.

Nevertheless, size reduction of pepsin CLEA and CLEL formulations failed to improve catalytic activity of the material, although to a less pronounced degree as compared to that of urease nano-CLEL. Some activity retention through nanonization was observed in case of CLEL formulation originally yielding near zero catalytic activity. Glutaraldehyde crosslinked CLEA was also nanonized to yield some activity improvement, while the most successful example of dextran polyaldehyde crosslinked formulations have suffered a decline in activity upon nanonization.

It can be generalized that small degree of crosslinking, specific to pepsin due to low availability of crosslinkable surface residues, results in much softer final product as compared to more CLEA/CLEL method friendly proteins described in the “crosslinked protein nanoaggregates” project [16]. Therefore, even the method of homogenization under milder absolute ethanol

medium conditions, which proved very useful while working with softer formulations (Zakharyuta, A., PhD Thesis, Nanosized Crosslinked Protein Aggregates (nano-CLPA)), appears too harsh in the case of pepsin formulations. It follows to reason that these formulations are irreversibly damaged by the shear force necessary for successful nanonization. Improvement of crosslinking degree of pepsin CLEA formulations could provide the necessary mechanical integrity for further nanonization trials. Slight improvement in catalytic activity of some initially less successful CLEA/CLEL formulations through nanonization, might be explained due to release, and therefore improved surface availability, of catalytically preserved units within the crosslinked protein bulk during distortion of the macroscopic structure.

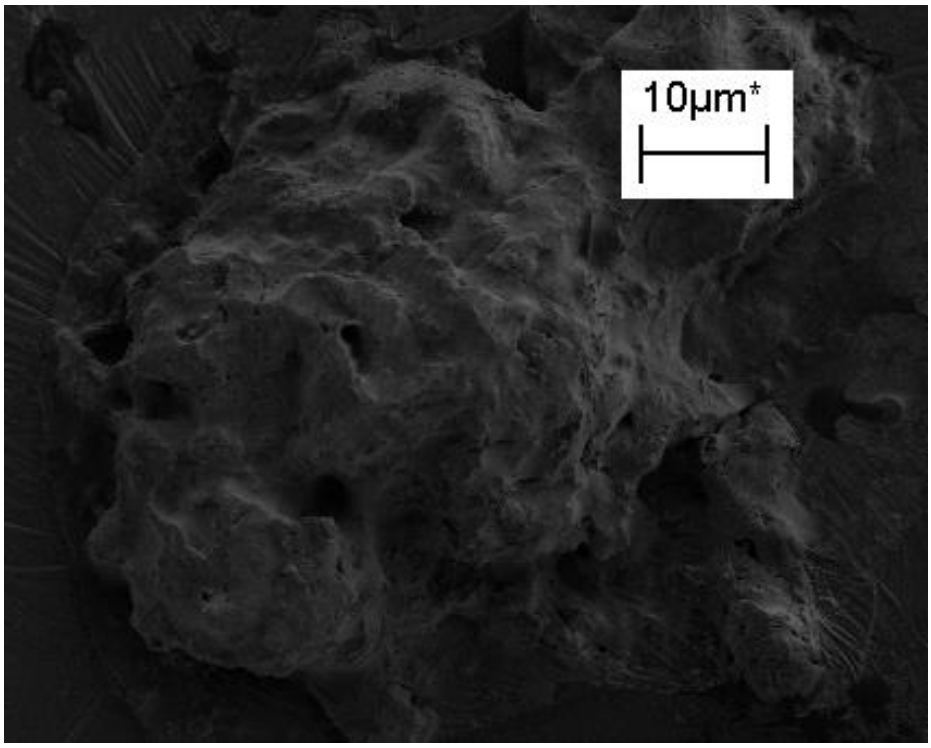


Figure 3-7 SEM imagery of crosslinked pepsin lyophilizate (CLEL), presenting the morphology of micro particle units; 2.00 KX, EHT = 2.00 kV, WD = 8 mm, Secondary electron detector

A general morphology of homogenization product is observe on Figure 3-7. SEM results are illustrated on Figure 3-8 presenting the morphology and approximate size order of

heterogeneously nanosized cross linked urease lyophilizates and interior of the generated particles on Figure 3-9. The finely structured heterogeneouse size nano particles with the average size around 200 nm can be observed (Figure 3-8). The particles of similar morphology have been observed in the previous study (Zakharyuta, A., PhD Thesis, Nanosized Crosslinked Protein Aggregates (nano-CLPA)). Nevertheless, in the case of pepsin, as has been oberved in the case of urease, the majority of the material is of the appearence visualized on Figure 3-9. This morphology also reflects the interior of the finely structured particles demonstrated, thereof providing evidence of mechanical integrity loss of the major part of crosslinked material in the course of homogenization process. The similarity in failure to achieve structural integrity in both cases arises from low crosslinking degree of both urease and pepsin crosslinked formulations.

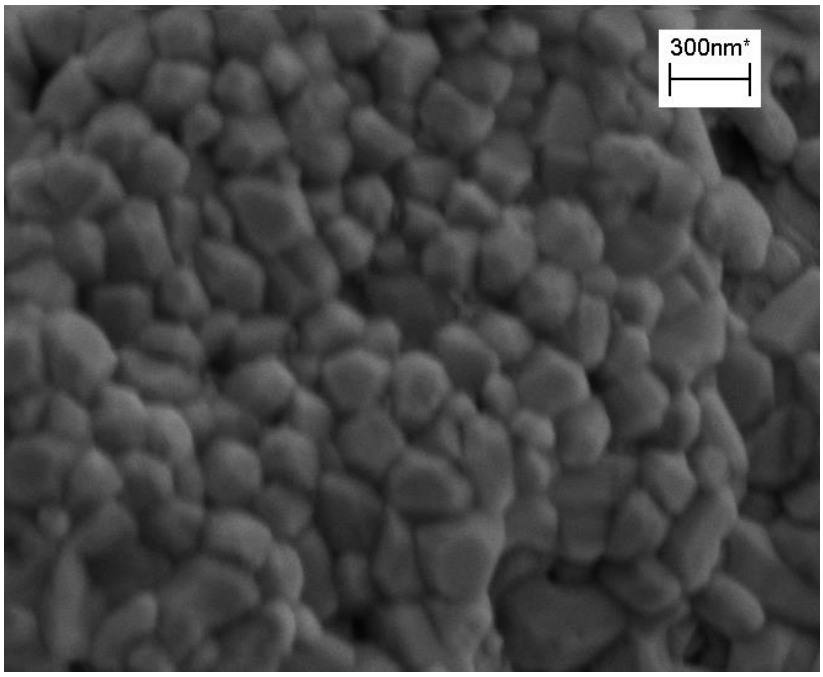


Figure 3-8 SEM imagery of nano crosslinked pepsin lyophilizate (nano CLEL), presenting the inferior morphology of nano particle units; 70.00 KX, EHT = 2.00 kV, WD = 8 mm, Secondary electron detector

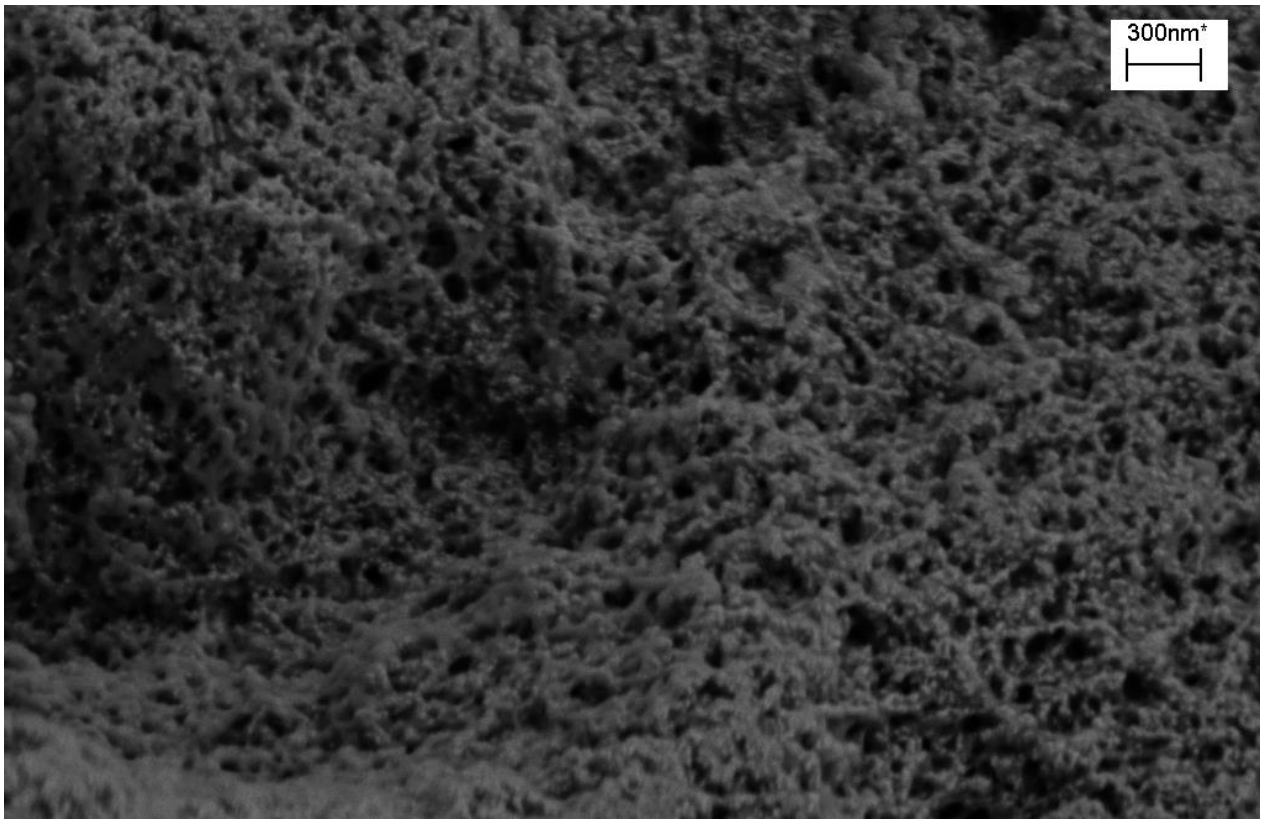


Figure 3-9 SEM imagery of nano crosslinked pepsin lyophilizate (nano CLEL), presenting the interior morphology of nano particle units; 70.00 KX, EHT = 2.00 kV, WD = 8 mm, Secondary electron detector

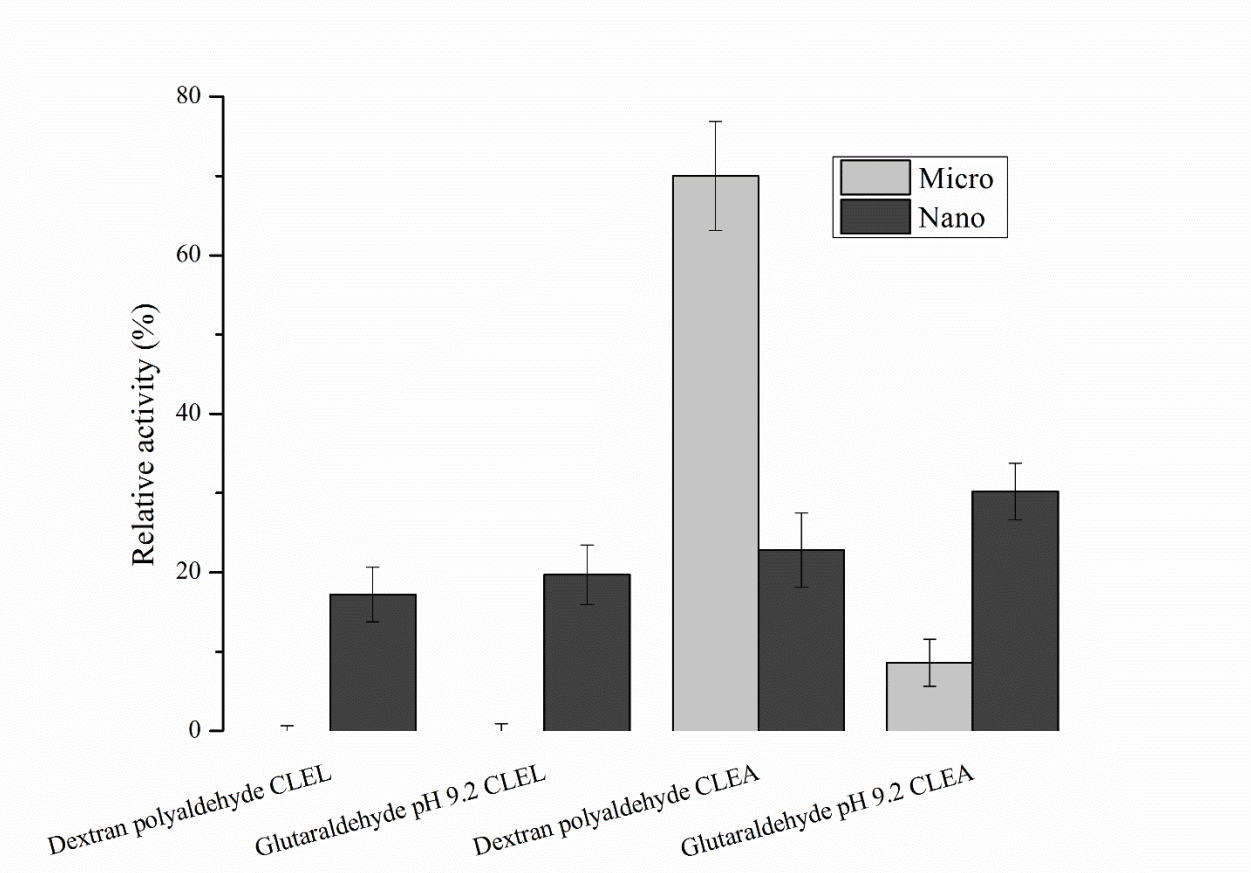


Figure 3-10 Catalytic activity comparison of pepsin CLEA/CLEL and nano CLEA/CLEL in relation to crosslinking reagent effect (aggregation/crosslinking medium-isopropanol)

3.5 Concluding Remarks

Most efficient aggregation and catalytic activity yields were obtained in case of utilization of isopropanol as aggregation medium. Not surprisingly, the problematic step appeared to be covalent crosslinking of pepsin, due to availability of only one lysine residue for cross link formation. The satisfactory solution was achieved through incorporation of dextran polyaldehyde as the cross linking reagent, and the use of the subzero crosslinking temperatures. Plausible results obtained from this optimum formulation may be facilitated by incorporation of surface hydroxyl groups into overall cross linking species. Formation of the first catalytically competent pepsin CLEA has been thereof achieved.

CHAPTER 4 Conclusion

Protein catalysts have had been widely utilized for a great range of industrial, analytical and biomedical applications for several decades, whereas the applicability of native protein formulations is limited and challenged by a number of factors including mechanical and chemical stability under harsh reaction conditions differing from those acceptable for a native protein due to its source and native specifications. In order to overcome these drawbacks and to enhance the enzymatic and catalytic properties of native proteins, the different immobilization techniques were developed to improve the protein stability under conditions varying from the native proteins optimum but necessary for a given application, such as temperature, pH, ionic strength, organic solvent etc. Furthermore, immobilization has been shown as an effective way to manipulate mechanical and enzymatic properties of native proteins that results in increased shelf life and provide reusability of the catalyst, while retaining catalytic activity of the enzyme in a proper way. The topic of this thesis forms a part of TÜBİTAK 1001 project no 111M680 "Crosslinked Protein Nanoaggregates" [16]. The main motivation of this project is to address challenges arising in micron and higher size heterogeneous catalyst systems, such as mass transport limitations, reduced access to catalytic site and etc., through the conventional and further optimized CLEA methodology via limiting the particle size to the nanoscale.

As described in Chapter 2, the conventional CLEA methods have failed to facilitate effective urease formulation. The results show us that low aggregation and crosslinking yields of urease CLEA led to statistically insignificant catalytic activity, compared with native one. The reason behind the poor catalytic activity of urease CLEA were well explained by the fact that urease displayed extremely poor protein precipitability which prevented protein stabilization and CLEA formation. Moreover, urease displayed unusual surface properties, i.e.; the conformation of functional groups required for crosslinking are pointing towards the hydrophobic core of urease and these conformations are not suitable to establish a proper interaction with the crosslinker. In order to facilitate originally impaired precipitability, urease was partially cross linked through the solution-phase crosslink-assisted aggregation method (CLEA). Results although somewhat

improved in terms of final product formation yield, still yielded a maximum of about 14% relative catalytic activity at its best formulation. The solution to the problem was finally achieved by enforcing protein aggregation via flash-freezing and lyophilization as opposed to routine precipitation from aqueous media by introduction of a suitable anti-solvent or salt. In this way, protein recovery as an aggregate was near-quantitative. Moreover, the subsequent crosslinking of the lyophilizate (yielding a CLEL) in a suitable anti-solvent bearing the crosslinker led to a much improved crosslinking yield and catalytic activity, yielding about 270% in the best obtained CLEL formulation. Therefore, the production of the first crosslinking mediated immobilization of urease has been established as urease CLEL.

Applicability of urease CLEL in organic synthesis has been demonstrated, resulting in modest reaction yields while still significantly facilitating the reaction catalyst. By prolonging reaction periods and making use the enhanced thermal stability of immobilized urease formulation, by increasing reaction temperatures the synthesis yield could be further enhanced.

In Chapter 3, the problematic covalent crosslinking of pepsin was addressed by modification of conventional CLEA methods. Structural challenge of pepsin towards conventional surface modification lies within appearance of only one lysine group, necessary for covalent cross link formation. Given that the formation of a crosslinked mass would demand two and at times three reactive groups per monomer, it was not surprising that pepsin was relatively unresponsive to all crosslinking attempts mediated by surface amino groups. This drawback has been bypassed by appropriate choice of a rather bulky dextran polyaldehyde reagent, which in some cases combined with the use of the subzero crosslinking temperatures resulted in much improved formation yields and has allowed the relative catalytic activity retention of about 80% in the best obtained formulation. It followed to reason that a combination of the large bulk and low temperature could have permitted the close interaction of protein and crosslinker, leading to secondary benefits such as crosslinker interactions with surface hydroxyl groups, and mechanical fastening of the structures to one another by way of encapsulation and intertwining. These secondary effects appeared to have tipped the scale, permitting the formation of the first ever catalytically competent pepsin CLEA. The successful production of first pepsin CLEA in literature was achieved.

Nanonization trials for both, urease and pepsin, CLEA/CLEL have been met with difficulties. While effective generation of nano particles has been achieved in both cases, resultant nano materials failed to achieve the expected increase in relative catalytic activity. Furthermore, significant decline in catalytic activity of urease nano CLEL was observed. It follows to reason that mechanical integrity of both materials was not sufficient to withstand the homogenization conditions dictated by nano particle generation requirements, which is readily rationalized by limited number of covalent crosslinks formed due to structural restrictions of both proteins. Further improvement of cross linking method, and thereof facilitation of mechanical stability necessary for harsh nanonization conditions, lies within the scope of future work.

It follows to reason that the novel immobilization protocols presented herein will add to the general knowledge base and potentially serve as a crucial alternative method in the case of other user-unfriendly protein systems important to industry. These could also be utilized to prompt greener processes, such as the enzymatic or semi-enzymatic syntheses of industrially important commodity compounds.

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Mar. 2014 "Chemical Reactions Facilitated by Crosslinked Urease Aggregates", 247th ACS National Meeting & Exposition, Dallas, Texas, USA

Jan. 2014 "Crosslinked Urease Nanoaggregates and Their Potential Utility in Chemical Reactions", 1st International Symposium on Nanoparticles/Nanomaterials and Applications, Lisbon, Portugal

Aug. 2013 "Crosslinked Urease Nanoaggregates and Their Potential Utility in Chemical Reactions", IUPAC 44th World Chemistry Congress, Istanbul, Turkey

Sept. 2011 "Internalization of nano crosslinked protein aggregates by HeLa cells", Workshop on Biomaterials and Their Interactions with Biological and Model Membranes, Barcelona, Spain

UNSUBMITTED WORK

Akkaş, T., Taralp, A., Zakharyuta,A. *A Practical Approach to Prepare CLEAs Using Poorly-Precipitatable Enzymes (Urease)*

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Sept. 2010 – Feb.2015	Sabanci University Faculty of Engineering and Natural Sciences full tuition fee exemption and housing support
Sept. 2012	R&D Project Market, Drug&Pharmacy Division 3rd prize, "Crosslinked Protein Nanoaggregates", Alpay Taralp, Anastasia Zakharyuta
Dec. 2012	Turkey Innovation Week, Most Innovative R&D Project prize, "Crosslinked Protein Nanoaggregates", Alpay Taralp, Anastasia Zakharyuta
Sept. 2012 – Feb. 2013	Erasmus Socrates European Union education and training programme, Belgium Universiteit Hasselt full scholarship from Turkish National Agency
2008	Istanbul Technical University, Chemistry Department, Spring Term Graduates, 6^{th} place
2004	Avni Akyol Private High School, 2004 graduates, 2^{nd} place
2002 – 2003	Avni Akyol Private High School, 1^{st} place