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# Highly biocompatible enzyme aggregates crosslinked by L-lysine

## [L-Lizin amino asidi ile çaprazbağlı yüksek biyouyumlu enzim agregatları]

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**ABSTRACT**

**Aim:** The purpose of the work is the use of L-Lysine amino acid cross-linker in the achievement of alternative and compatible enzyme aggregate. Cross-linked enzyme aggregates (CLEAs) were prepared from several enzymes (glucose oxidase, peroxidase and urease) by precipitation and subsequent cross-linking using glutaraldehyde, 1,8-octanediamine and L-Lysine.

**Material and Methods:** The effects of cross-linking agents on CLEAs activity were investigated and immobilized enzymes were characterized. The initial enzyme concentration was constant as $4x10^{-3}$ mg/ml. BSA were used as precipitant in CLEA's method as described in our previously study.

**Results:** The concentration of cross-linkers were 2% for glutaraldehyde and 1,8-octanediamine and 4% for L-Lysine. Activities of both free and immobilised enzymes were obtained by measuring the amount of substrate conversion, spectrophotometrically. Kinetic parameters of native and immobilised enzyme were calculated by using Lineweaver-Burk plots.

**Conclusion:** L-Lysine was applied successfully as a cross-linker for the formation of CLEA's.

**Key words**: Cross-linked enzyme aggregates, Glucose Oxidase, Biocompatibility, L-Lysine.

**Conflict of interest:** The authors do not have a conflict of interest.

**ÖZET**

**Amaç:** Çalışmanın amacı lizin amino asit çapraz bağlayıcısının alternatif ve biyouyumlu enzim aggregat elde edilmesinde kullanımıdır. Sunulan çalışma kapsamında, farklı enzimler (glukoz oksidaz, peroksidaz ve üreaz), çöktürme işlemi ve ardından farklı çapraz bağlayıcılar (glutaraldehid, 1,8-oktandiamin ve lizin) ile çapraz bağlanarak, çağraz bağlı enzim agregatları (CLEA) hazırlanmıştır.

**Gereç ve Yöntemler:** Çalışmada çapraz bağlayıcı ajanların, CLEA'ların aktivitesi üzerine etkisi incelenmiş ve immobilize enzimler karakterize edilmişlerdir. Başlangıç enzim konsantrasyonları, $4x10^{-3}$ mg/ml olmak üzere sabit tutulmuştur. BSA daha önceki çalışmamızda tanımlandığı gibi CLEA'ların hazırlanmasında çöktürücü olarak kullanılmıştır.

**Bulgular:** Denemelerde glutaraldehid ve 1,8-oktandiamin %2 ve L-lizin %4 oranlarında kullanılmıştır. Serbest ve immobilize enzim aktiviteleri spektrofotometrik olarak subtrat dönüşüm miktarları ölçümü ile elde edilmiştir. Doğal ve immobilize enzimlerin kinetik parametreleri Lineweaver-Burk eğrisi kullanılarak hesaplanmıştır.

**Sonuç:** L-lizin aminoasidi, çapraz bağlayıcı olarak CLEA'ların hazırlanmasında başarıyla uygulanmıştır.

**Anahtar kelimeler**: Çapraz bağlı enzim agregatları, Glıkoz oksidaz, Biyouyumluluk, L-lizin

**Çıkar çatışması:** Yazarların çıkar çatışması yoktur..

## Introduction

Investigations about enzymes were devoted to the improvement of various carrier bound immobilised enzymes with the aim of facilitating their use in continuous processes. Enzyme immobilisation can accomplish developed enzyme performance such as activity, stability and selectivity. Successful immobilisation depends on selection a suitable carrier, conditions (pH, temperature, medium) and enzyme. Because of this, the design of carrier-bound immobilised enzymes relies largely on laborious and time-consuming trial and error experiments [1]. So, there is a new interest in carrier-free immobilised enzymes such as cross-linked enzyme aggregates (CLEA) [2].

CLEAs were added to the immobilised enzymes techniques more recently [2]. By altering properties that affect the proximity of soluble enzyme molecules, they can be made to form physical aggregates after cross-linking. For example, it is possible to form aggregates by changing the hydration state of enzyme molecules or changing the electrostatic constant of the solution by adding appropriate aggregations agents [3]. Enzymes molecules were precipitated as insoluble aggregates with native enzyme conformation and these insoluble aggregates can be cross-linked by bifunctional cross-linkers [2].

CLEAs can be prepared with greater mechanical stability and tailor-made size. In principle, CLEAs are applicable to any reaction system, reactor configuration and reaction medium. Recent studies enclose the development of CLEAs of abroad range of enzymes, size control, new aggregations methods, new precipitants and new cross linkers to construct a flexible technology platform for designing robust CLEAs for broad applications [3].

In this study glucose oxidase, peroxidase and urease were cross-linked by using an amino acid L-lysine in the presence of BSA as the proteic feeder in the achievement of alternative and compatible enzyme aggregates. The enzymes were also cross-linked using glutaraldehyde, 1,8-diaminooctane, respectively (Figure 1). The prepared cross-linked enzyme aggregates were compared on account of activity.

## Materials and Methods

### *Chemicals*

Glucose Oxidase (GOD) from *Aspergillus niger* (E.C.232-601-0), peroxidase (POD) from *Horseradish* (E.C. 232-668-6), urease (E.C.3.5.1.5), glutaraldehyde (GA), 1,8-diaminooctane, L-lysine, bovine serum albumin (BSA), pyrogallol, glucose and urea were purchased from Sigma. All other reagents used were of analytical grade.

### *Preparation of enzyme aggregates*

Chemical aggregates of three enzymes, namely glucose oxidase, peroxidase, and urease were prepared as reported earlier [4]. The enzymes were aggregated in the presence of BSA as a proteic feeder. Enzyme aggregates were prepared by dissolving $4x10^{-3}$ mg/ml enzyme and 5 mg BSA in 0.8 ml of acetate buffer (0.1 M, pH 5.0).

### *Cross-linking of enzyme aggregates*

### *Cross-linking with glutaraldehyde*

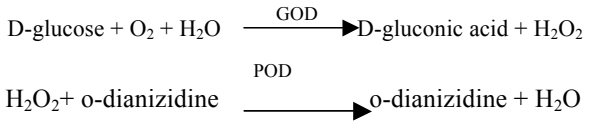
The enzyme aggregates was mixed with glutaraldehyde (concentration is 2%) and final volume was made to 1 ml with distilled water. After incubation at 25˚C for 2 h (with constant stirring for the first 15 min), crosslinked enzyme aggregates were collected by centrifugation for 15 min at 4˚C and homogenized with a glass rod. The reaction mixture was left overnight at 4˚C. Crosslinked enzyme aggregates were collected by centrifugation and washed extensively with distilled water until the supernatant was free of protein [4].

### *Cross-linking with 1,8-octanediamine or L-lysine*

Physically aggregated enzymes were subjected to chemical cross-linking with different crosslinkers using 1,8-octanediamine and L-Lysine. The hydroxyl groups on enzymes were oxidized by $NaIO_4$ to obtain aldehyde groups. The oxidation was performed in $2x10^{-2}$ mmol/ml $NaIO_4$ solution for 1 h at room temperature which was containing protein feeder (BSA) and enzyme solution [5,6]. Then, cross linkers (1,8-diaminooctane and /or L-lysine) were added to the reaction media whose concentrations were ranged from 2% to 6%. The enzyme aggregates were incubated at 25˚C for 2 h (with constant stirring-7000 rpm for the first 15 min) then collected by centrifugation for 15 min at 4˚C and washed extensively with distilled water until the supernatant was free of protein. The concentrations of 1,8-diaminooctane and L-lysine was selected as % 4 volume/volume after the pre-experiments.

### *Free enzyme and GOD aggregates assay measurement*

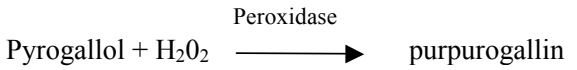
Activities of both free and immobilised GOD were obtained by measuring the amount of hydrogen peroxide formed from glucose conversion, spectrophotometrically [7]. 2.5 ml of POD (1.5 mg) and o-dianisidine (3.3 mg) mixture was added into 50 ml of 0.1 mM phosphate buffer (pH 7.0). This solution was incubated for 10 min at 25˚C. 100 µl of sample which was obtained by the oxidation of D-glucose by GOD, was added to the assay mixture. After 10 min, 1.5 ml of sulphuric acid solution (30%) was added to this mixture to stabilize the colour formed. The enzyme activity was measured spectrophotometrically at 525 nm [7].




One unit of glucose oxidase is defined as the amount of enzyme which oxidizes 1 μM of β-D-glucose to D-gluconic acid and hydrogen peroxide per min at 25˚C and pH 7.0.

### *Free enzyme and peroxidase aggregates assay measurement*

The method of assay measures the oxidation of pyrogallol to purpurogallin by peroxidase. The reaction was monitored spectrophotometrically at 420 nm and at 20˚C.




The activity measurment of native and aggregated peroxidase enzymes were performed one by one. 0.1 ml of native POD/0.1 ml of CLEA of POD were added to 2.4 ml of an assay mixture containing 12.8 mmol/ L pyrogallol and 0.1 mol / L phosphate buffer. The reactions were started with the addition of 0.5 ml $H_2O_2$ solution (5.0 mmol / L). The assay method measures the oxidation of pyrogallol to purpurogallin by peroxidase. The reaction was monitored spectrophotometrically at 420 nm and at 20°C. The reaction of the CLEA of POD was tested at time intervals from 1 to 3 min [8]. One unit of peroxidase was defined as the amount of enzyme which is turned 1mg of pirogallol to 1 mg purpurogallin and hydrogen peroxide per 20 seconds at 25˚C and pH 6.0.

### *Free enzyme and urease aggregates assay measurement*

Native and CLEA of urease were added into 20 ml pH 7 phosphate buffer (1/15 M $NaH_2PO_4$ + 1/15 M $Na_2HPO_4$) containing 1.5 mg/ml (25 mM) urea. Reaction was started in a sealed glass reactor which was stirred at 200 rpm at 25˚C. The concentration of ammonia liberated was determined by the Nessler ammonia assay method at 405 nm [9].One unit of urease was defined as the amount of enzyme which is turned urea to 1 μM ammonia at 25˚C and pH 7.0.

All of the experiments were repeated at least five times and the results were reported as the average of the experiments.

## Results and Discussions

In this study, CLEA's of glucose oxidase, peroxidase and urease were prepared with different crosslinkers (Figure 1) to investigate the effect of cross-linker type on CLEA's formation and activity.

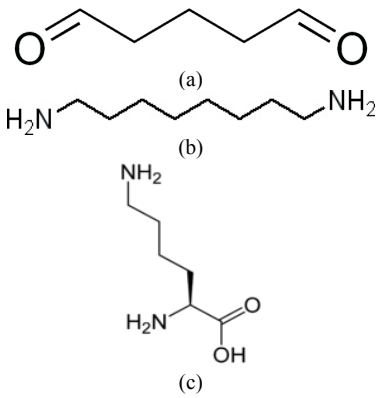



**Figure 1.** Crosslinkers which were used to prepare CLEAs (a) Glutaraldehyde; (b) 1,8-diaminooctane; (c) L-Lysine.

Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities. However, an inherent drawback is that glutaraldehyde and 1,8-diaminooctane are not biocompatible molecules and glutaraldehyde has also limited use because of its toxicity Therefore, we crosslinked the enzyme aggregates (GOD, POD and urease) with a biocompatible and non-toxic biomolecule L-lysine and compared with glutaraldehyde and 1,8-octanediamine crosslinked enzyme aggregates. L-Lysine is an amino acid with a pharmacological use much more specific than that of most other amino acids and is not used as a cross-linker in the immobilization process so far. L-Lysine amino acid and 1-8 diaminooctane are known as highly reactant diamine molecules.

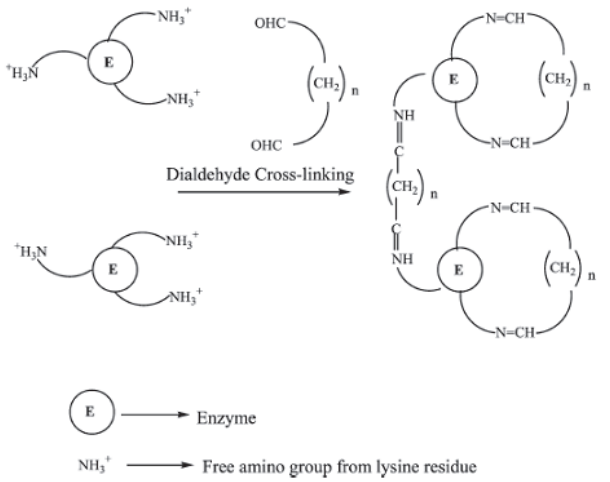



**Figure 2.** Schematic presentation of dialdehyde cross-linking.

Figure 2 shows the cross-linking reactions become via functional groups of residues of enzymes. The cross-linking reaction with glutaraldehyde occurs via reaction of the free amino groups of L-Lysine residues, on the surface of neighbouring enzyme molecules, with oligomers or polymers resulting from aldol condensations of glutaraldehyde. Figure 3 shows the oxidation reaction –OH groups of enzyme to aldehyde groups and cross-

linking reaction of enzyme with L-Lysine. To crosslink enzymes with L-Lysine/ 1,8-diaminooctane, –OH groups must be oxidized to yield aldehyde groups. Serine and threonine residues in proteins can be oxidized with periodate to yield aldehydes. Therefore –OH groups of enzyme were oxidized with $NaIO_4$ and then crosslinking reaction occurred by adding L-Lysine/ 1,8-diaminooctane to the solution.

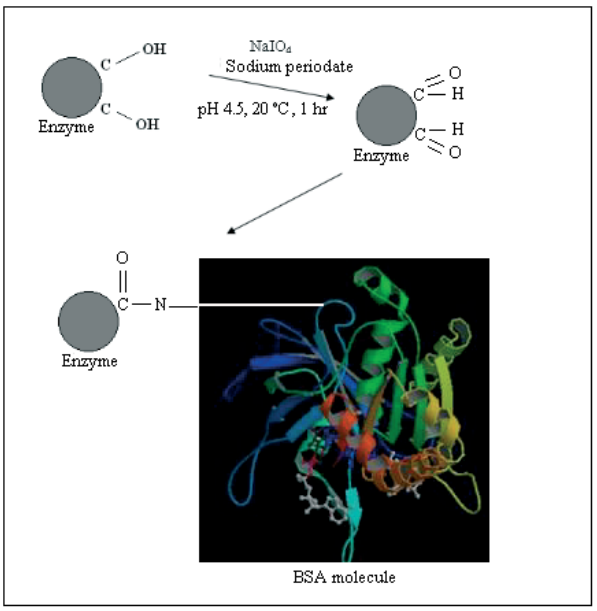



**Figure 3.** Schematic presentation of $NaIO_4$ oxidation and diamine cross-linking.
(XRay micrograph of BSA obtained from http://www.bioworld.com/productinfo/2_36_259/8615/BSA-Bovine-Serum-Albumin-A.html)

The immobilization efficiency values for all cross-linkers are shown in Figure 4. According to this figure there was no appreciable difference in the immobilization efficiency. But immobilization efficiency was a bit more when GA was used as crosslinker. The change trend in immobilization efficiency of the aggregates with 1,8-diaminooctane and L-Lysine were also very close. Because they both have the amine groups. The decrease in the immobilization efficiency with 1,8-diaminooctane and L-Lysine probably were caused by $NaIO_4$ oxidation. Because periodate oxidation causes local changes of structure in protein molecules, but does not grossly alter the conformation.

The cross linking reaction with gluraldehyde took place by free amine groups of L-Lysine residue on enzyme, however the reaction with 1,8- diaminooctane and L-lysine took place after oxidation of –OH groups of enzymes to aldehyde groups.

Figure 5 shows the enzyme activities of the aggregates corresponding to the each cross-linker and enzymes. According to Figure *5,* activity values decreased due to immobilization efficiency and also effected by using less amount of enzyme. Reduction in activity was more, in comparison with immobilization efficiency reduction.

As it can be seen from *Figure 5*, cross-linking with 1,8-diaminooctane and L-Lysine, the activity decreased

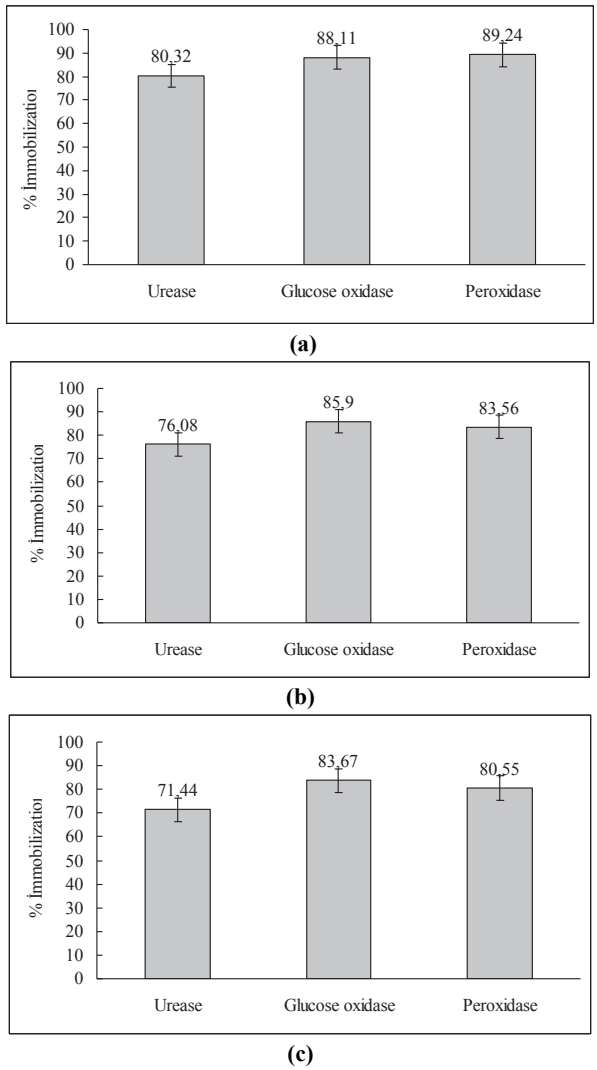
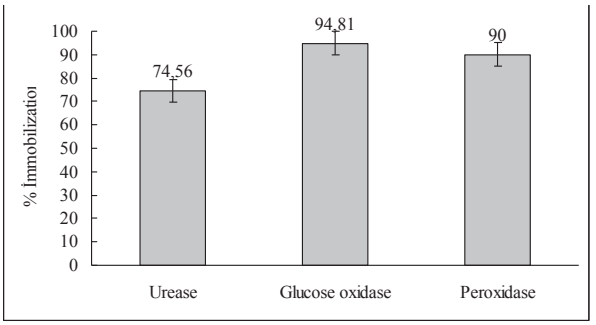



**Figure 4.** Effect of cross-linkers on immobilization efficiency a) glutaraldehyde; b) 1,8-diaminooctane; c) L-Lysine as a cross-linker (Enzyme concentration: $4x10^{-3}$mg/ml, BSA concentration: 5 mg, pH 5.0, 25ºC) All of the data are the average of the five experiments.

depend on increasing the numbers of -OH groups, molecular weights, and unfolding properties of enzymes. Because $NaIO_4$ oxidation caused to change three dimensional structure of enzyme molecules before using 1,8-diaminooctane and L-lysine. But still the activity values were % 50±10. These values were quite successful.

The effect of cross-linkers on the kinetic parameters, $K_m$ and $V_m$ were also calculated by Lineweaver-Burk plots. There was an appreciable difference for $K_m$ and $V_m$ values between free enzymes and enzyme aggreagates (Table 1). The possible reason of this difference could be based on the molecular deformation which had realized in course of cross-linking procedure. The cross-linking reactions affected the three dimensional structure of enzyme and functional groups.

The kinetic parameters of immobilised GOD enzyme were studied in the earlier experiments. As reported earlier [10] the saturation constant (Km) and the maximum reaction rate (Vm) of free enzyme were as 68.2 mM and 435 µmol

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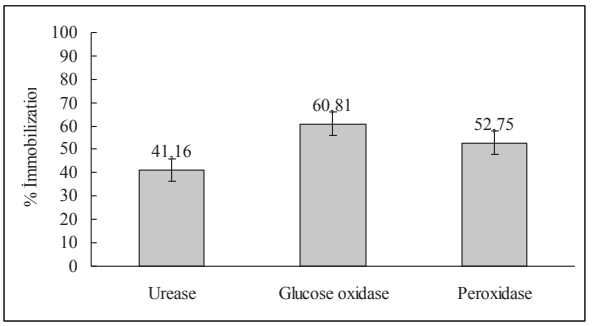


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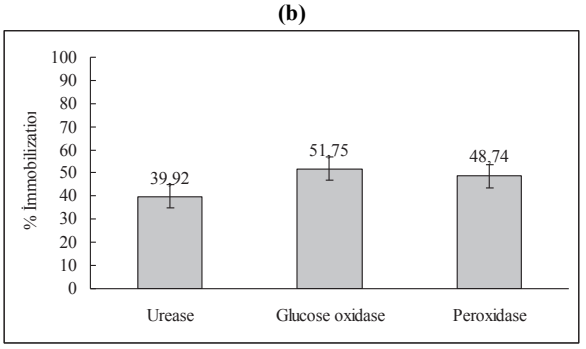



**(c)**

**Figure 5.** Effect of cross-linkers on enzyme activity a) glutaraldehyde; b) 1,8-diaminooctane; c) L-lysine as a cross-linker (Enzyme concentration: $4x10^{-3}$mg/ml, BSA concentration: 5 mg, pH 5.0, 25ºC) All of the data are the average of the five experiments.

$min^{-1}$ respectively. (Km) and (Vm) of immobilised GOD onto magnesium silicate were 259 mM and 217 µmol min-1. In general, $K_m$ values of immobilized enzymes are higher and $V_m$ values are lower than free enzymes because of steric hindrances and diffusion limitations. But, in present study, the differences of $K_m$ (substrate affinity) between free enzyme and CLEAs are low.

*Figure 6* indicates scanning electron micrographs (SEM) of different enzyme (GOD, POD, urease) aggregates by using glutaraldehyde as crosslinker. *Figure 7* shows the SEM of GOD aggregates prepared with (a) glutaraldehyde, (b) 1,8-diaminooctane and (c) L-lysine. The micrographs indicate a compact, amorph and ductile network structure. The GOD aggregates show spongy structure which is typical for CLEAs except L-Lysine. The controlling of aggregates size and its effect on the activity are very important. If the size of aggregates is too big, the enzyme molecules lose to react with the substrate molecules and decrease their activity.

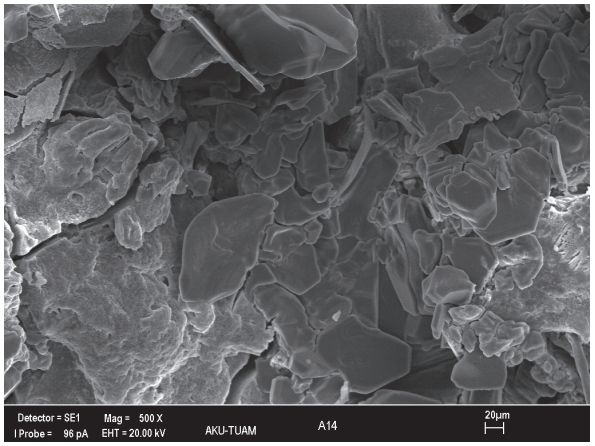
## Conclusion

The present study demonstrated a simple and successful routine to obtain robust and biocompatible GOD, POD and urease CLEAs by using L-Lysine as cross-linker and bovine serum albumin (BSA) as proteic feeder. CLEAs prepared with L-Lysine cross-linker was not exhibited enzyme activity as in the case of glutaraldehyde cross-linker but still the activity values were around 60 % compared whit that of free enzyme and these values can be regarded as successful. The strategy is extremely simple and may be of general use to produce rigid, stable and without uncompatible agents CLEAs, which can be utilized to enhance the enzymatic catalysis efficiency.

**Conflict of interest:** The authors do not have a conflict of interest.

**Table 1.** Kinetic parametres of free enzymes and enzyme aggregate

| | | $K_m$ (mM) | $V_m$ (mM.$min^{-1}$) |
|---|---|---|---|
| **GOD** | Free Enzyme | 7.9 | 66.29 |
| | Cross-linking with glutaraldehyde | 11.37 | 12.22 |
| | Cross-linking with 1,8-diaminooctane | 20.54 | 9.29 |
| | Cross-linking with L-Lysine | 24.88 | 7.34 |
| | | **$K_m$ (mmol/l)** | **$V_m$ (mmol/l.s)** |
| **POD** | Free Enzyme | 2.33 | 0.76 |
| | Cross-linking with glutaraldehyde | 33.4 | 0.127 |
| | Cross-linking with 1,8-diaminooctane | 36.27 | 0.10 |
| | Cross-linking with L-Lysine | 34.72 | 0.23 |
| | | **$K_m$ (µmol/l)** | **$V_m$ (µmol.$s^{-1}$). $10^{-4}$** |
| **UREASE** | Free Enzyme | 16.34 | 2.53 |
| | Cross-linking with glutaraldehyde | 22.17 | 1.89 |
| | Cross-linking with 1,8-diaminooctane | 27.53 | 1.04 |
| | Cross-linking with L-Lysine | 29.45 | 0.95 |

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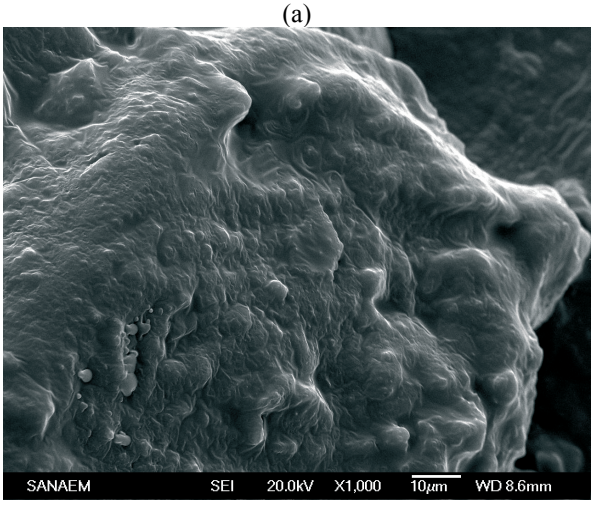


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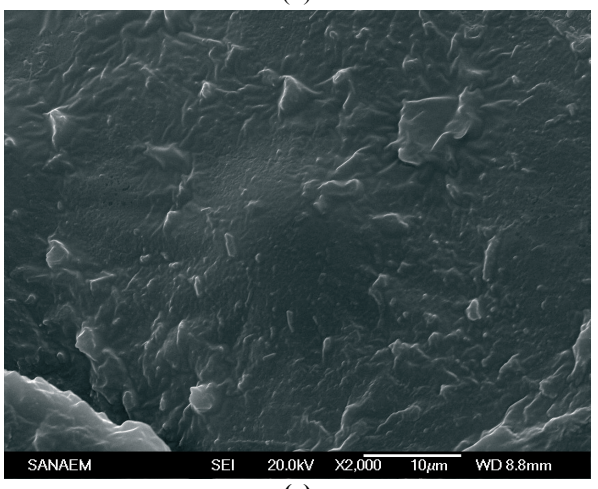
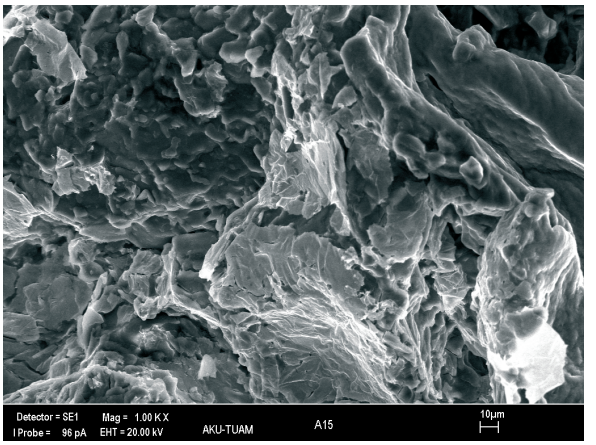



(c)

**Figure 6.** SEM photographs different aggregates with using Glutaraldehide. a) GOD; b) POD c) Urease

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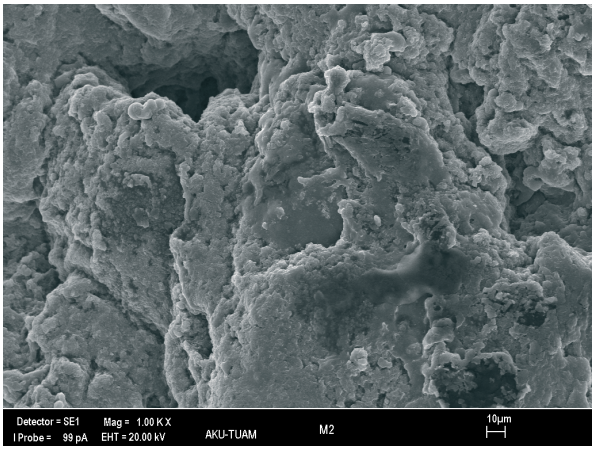


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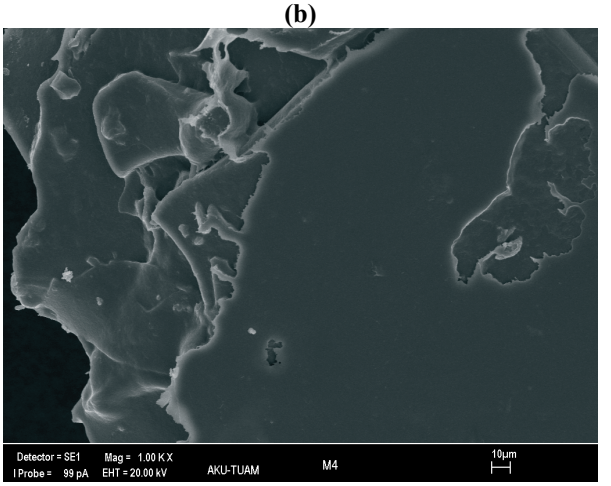


**(c)**

**Figure 7.** SEM photographs GOD enzyme aggregates with different cross-linkers; a) with glutaraldehyde; b) with 1,8-diaminooctane; c) with L-lysine.

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