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Glutaraldehyde in Protein Immobilization

A Versatile Reagent

Lorena Betancor, Fernando López-Gallego, Noelia Alonso-Morales, Gisella Dellamora, Cesar Mateo, Roberto Fernandez-Lafuente, and Jose M. Guisan

Summary

The use of glutaraldehyde and supports containing primary amino groups is one of the most frequently used techniques for enzyme immobilization. However, glutaraldehyde is a very versatile reagent. Using low-ionic strength, the cationic nature of the surface permits the rapid ionic immobilization of the proteins. There are two different possibilities: (1) activate the support and immobilize the enzyme in a glutaraldehyde-activated support (in this case the immobilization is promoted by ionic exchange) or (2) adsorb the proteins on the aminated supports and treat the immobilized preparation with glutaraldehyde to cross-link both the enzyme and the support. Both alternatives have advantages and drawbacks that will be discussed on this chapter.

Key Words: Support–enzyme crosslinking; ionic adsorption; multipoint covalent attachment; enzyme stabilization; multipoint covalent attachment.

1. Introdution

Covalent immobilization of enzymes by means of glutaraldehyde chemistry is one of the most frequently used technologies for enzyme immobilization. There are several ways of using glutaraldehyde for this purpose, such as the immobilization of enzymes on supports previously activated with glutaraldehyde (*see* **Fig. 1**) ***(1–8)*** or the treatment with glutaraldehyde of proteins adsorbed on supports having primary amino groups (*see* **Fig. 2**) ***(9–10)***.

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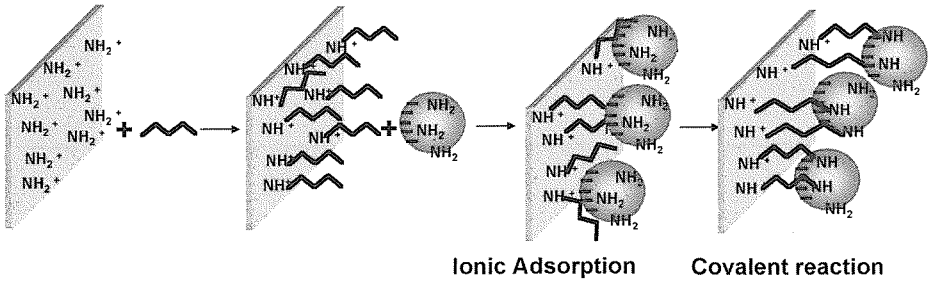

Fig. 1. Protein immobilization on aminated supports pre-activated with glutaraldehyde.

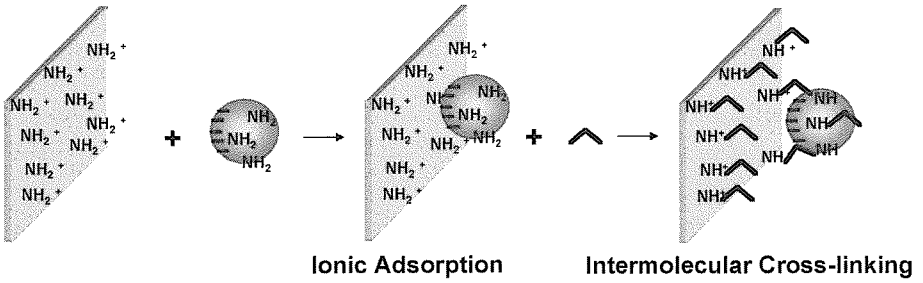

Fig. 2. Cross-linking with glutaraldehyde of proteins ionically adsorbed onto aminated cationic supports.

1.1. Protein Immobilization on Aminated Supports Preactivated With Glutaraldehyde

The immobilization of proteins on glutaraldehyde preactivated supports is quite simple and efficient, and in some instances even permits the improvement of enzyme stability by multipoint or multisubunit immobilization *(7,8)*.

These supports are made by derivatization with glutaraldehyde of a matrix that originally must contain primary amino groups. This means that below each glutaraldehyde molecule, there are one or two amino groups (e.g., in the case of epoxy or aldehyde supports activated with ethylenediamine) *(11)* that can confer some ionic exchanger features to the support. Such supports can be considered heterofunctional matrixes in a manner similar to the recently described heterofunctional epoxy supports *(12–15)*. And they take advantage of the possibility that an ionic exchange of the proteins occurs on the support before the covalent reaction is permitted (*see* **Fig. 1**) *(16)*.

1.2. Cross-Linking With Glutaraldehyde of Proteins Ionically Adsorbed Onto Aminated Cationic Supports

Another strategy for immobilizing proteins using the glutaraldehyde chemistry is to treat proteins previously adsorbed on cationic supports containing primary amino groups with glutaraldehyde. The enzyme is first ionically adsorbed onto the ionic exchanger support and then treated with glutaraldehyde under mild condi-

tions. All the primary amino
with one molecule of glutara
occur. It has been shown tha
amino group) may promote
conditions *(17)*. It has also
using the glutaraldehyde gro
port *(18)*.

2. Materials

1. Aminated supports: M
   Aminopropil-CPG (Mil
   Aldrich St. Luis, MO), S
2. Immobilization buffer: 2
3. 25% (v/v) Glutaraldehyd

3. Methods

3.1. Activation of Aminate

1. Prepare a 15% glutaralde
   the pH to 7.0 (*see* **Note 1**
2. Suspend 10 mL of an am
   prepared in **step 1** (*see* **N**
3. Gently stir the suspensio
   wash the suspension wit
   oughly with distilled wat

3.2. Immobilization of Enz

1. Suspend 10 g of the of gl
   immobilization buffer (*se*
2. Gently stir at 25°C.
3. Withdraw aliquots from
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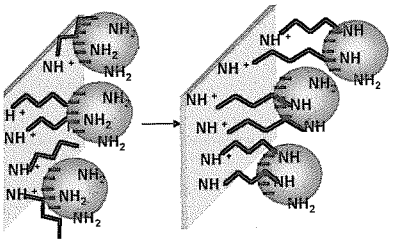
4. Vacuum filter the derivati

3.3. Adsorption of Enzyme

1. Suspend 10 g of an aminat
   immobilization buffer (*se*
2. Gently stir at 25°C.
3. Withdraw aliquots from s
   tivity until total adsorptio
4. Wash the adsorbed enzym

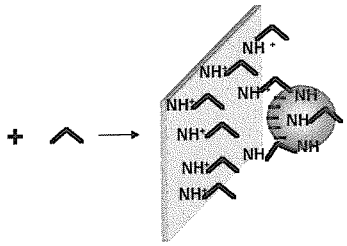
3.4. Cross-Linking of Adsor

1. Prepare a 0.5% (v/v) glutar
   pH 7.0.
2. Suspend 1 wet g of the ad
   of the glutaraldehyde solu
3. Gently stir for 1 h at 25°C



Isorption Covalent reaction

orts pre-activated with glutaraldehyde.



Intermolecular Cross-linking

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Supports Preactivated With

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ao groups (e.g., in the case of epoxy
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Such supports can be considered
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they take advantage of the possibil-
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f Proteins Ionically Adsorbed

using the glutaraldehyde chemistry
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ie is first ionically adsorbed onto the
h glutaraldehyde under mild condi-

tions. All the primary amino groups of the enzyme and support should be activated with one molecule of glutaraldehyde; the support–enzyme cross-linking may then occur. It has been shown that these glutaraldehyde groups (just one molecule per amino group) may promote intense cross-linking under a broad range of reaction conditions *(17)*. It has also been shown that a similar result might be obtained using the glutaraldehyde groups of the activated enzyme and those from the support *(18)*.

2. Materials

1. Aminated supports: MANAE agarose (Hispanagar S.A., Burgos, Spain), Aminopropil-CPG (Millipore billerica, MA), Aminoethyl agarose (Sigma-Aldrich St. Luis, MO), Sepabeads® EC-EA (Resindion SRL, Milan, Italy).
2. Immobilization buffer: 25 m*M* sodium potassium, pH 7.0.
3. 25% (v/v) Glutaraldehyde solution (Fluka, Switzerland).

3. Methods

3.1. Activation of Aminated Supports Activated With Glutaraldehyde

1. Prepare a 15% glutaraldehyde solution in 200 m*M* phosphate buffer and adjust the pH to 7.0 (*see* **Note 1**).
2. Suspend 10 mL of an aminated support in 20 mL of the glutaraldehyde solution prepared in **step 1** (*see* **Note 1**).
3. Gently stir the suspension for 15 h at 25°C (*see* **Note 2**). Filter and thoroughly wash the suspension with 5 vol 25 m*M* sodium phosphate buffer. Rinse thoroughly with distilled water. Store the gel at 4°C and use within 24 h.

3.2. Immobilization of Enzymes on Glutaraldehyde-Activated Supports

1. Suspend 10 g of the of glutaraldehyde support in 20 mL of enzymatic solution in immobilization buffer (*see* **Note 5**).
2. Gently stir at 25°C.
3. Withdraw aliquots from suspensions and supernatant and assay their catalytic activity. The frequency of sampling must be established for each immobilization.
4. Vacuum filter the derivative and wash thoroughly with distilled water.

3.3. Adsorption of Enzymes Onto Aminated Supports

1. Suspend 10 g of an aminated support in 20 mL of enzymatic solution prepared in immobilization buffer (*see* **Note 3**).
2. Gently stir at 25°C.
3. Withdraw aliquots from suspension and supernatant and assay their catalytic activity until total adsorption of the enzyme.
4. Wash the adsorbed enzyme thoroughly with distilled water and filter to dryness.

3.4. Cross-Linking of Adsorbed Derivatives With Glutaraldehyde

1. Prepare a 0.5% (v/v) glutaraldehyde solution (in 25 m*M* sodium phosphate buffer, pH 7.0.
2. Suspend 1 wet g of the adsorbed enzyme (*see* **Subheading 3.3., step 3**) in 4 mL of the glutaraldehyde solution.
3. Gently stir for 1 h at 25°C.

4. Filter and wash the modified immobilized enzyme thoroughly with 25 m*M* sodium phosphate buffer, pH 7.0, to remove the excess of glutaraldehyde. Filter to eliminate inter-particle water.
5. Keep for 20 h at 25°C and then store at 4°C (*see* **Note 4**).

3.5. Desorption of Noncovalently Immobilized Proteins on the Support

1. To check if the cross-linking has been successful, resuspend 0.5 g of derivative in 2.5 mL of 1 *M* sodium phosphate buffer, pH 7.0. Gently stir for 30 min at 20°C.
2. Assay the activity from the suspension and supernatant after the desorption process (*see* **Note 6**).

3.6. Immobilization–Stabilization of Glucose Oxidase Onto MANAE Agarose Preactivated With Glutaraldehyde

1. Prepare an enzymatic solution containing 13 U/mL 25 m*M* sodium phosphate buffer, pH 7.0.
2. Assay the catalytic activity of the enzymatic solution previously described. Add 10 g of MANAE agarose activated with glutaraldehyde to 20 mL of the glucose oxidase (GOX) solution and assay the enzyme activity of both the suspension and supernatant after 30 min at 25 C. If any activity remains in the supernatant stir the suspension for an additional 30 min under the same conditions. Repeat for the enzyme assays (*see* **Fig. 3**).
3. Filter and thoroughly wash the derivative with distilled water.
4. Evaluate the covalent immobilization as described previously (*see* **Subheading 3.5.**).
5. This preparation is more thermostable than the soluble enzyme (*see* **Fig. 4**).

3.7. Immobilization–Stabilization of D-Amino Acid Oxidase by Adsorption on Sepabeads™ EA-EC Plus Further Glutaraldehyde Treatment

1. Prepare a solution of D-amino acid occidase (DAAO) in 25 m*M* sodium phosphate, pH 7.0.
2. Assay the catalytic activity of this solution. Add 10 g of EC-EA to 20 mL of the previous DAAO solution. Measure the enzyme activity of both the suspension and supernatant after 30 min at 25°C.
3. Filter the derivative.
4. Add 1 g of derivative to 4 mL of 0.5% glutaraldehyde solution, pH 7.0. Gently stir for 1 h at 25°C.
5. Filter and wash suspension with the excess 25 m*M* sodium phosphate, pH 7.0. Incubate for 18 h at 25°C.
6. This derivative is more thermostable than the soluble enzyme (*see* **Fig. 5**).

3.8. Immobilization–Stabilization of Glutaryl Acylase by Adsorption on Sepabeads EA-EC Plus Further Glutaraldehyde Treatment

1. Prepare an enzymatic solution by mixing 5 mL of a commercial glutaryl acylase (GA) (Roche) with 20 mL of 25 m*M* potassium phosphate buffer, pH 7.0.
2. Assay the catalytic activity of the enzymatic solution previously described. Add 10 g of MANAE agarose to 20 mL of the GA solution and assay the enzyme activity of both the suspension and supernatant for 30 min at 25°C.

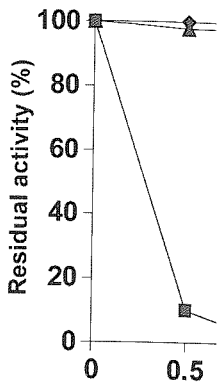

Fig. 3. Immobilization course
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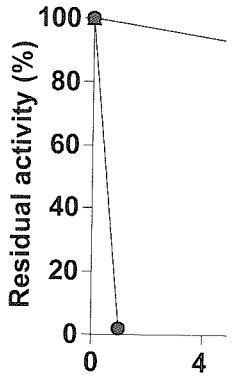

Fig. 4. Thermal stability of dif
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3. Filter the derivative. Add 1 g
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4. Filter and wash the suspensi
Incubate for 18 h at 25°C.
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see **Note 4**).

:ed Proteins on the Support

ful, resuspend 0.5 g of derivative in
7.0. Gently stir for 30 min at 20°C.
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ɜ Oxidase Onto MANAE

U/mL 25 m*M* sodium phosphate

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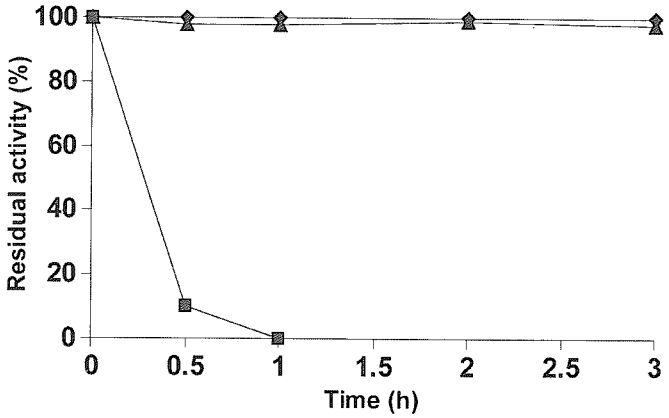

Fig. 3. Immobilization course of GOX on MANAE agarose activated with glutaraldehyde. Rhombus, control GOX solution; squares, supernatant, triangles, suspension. More details are described in **Subheading 3.6.**

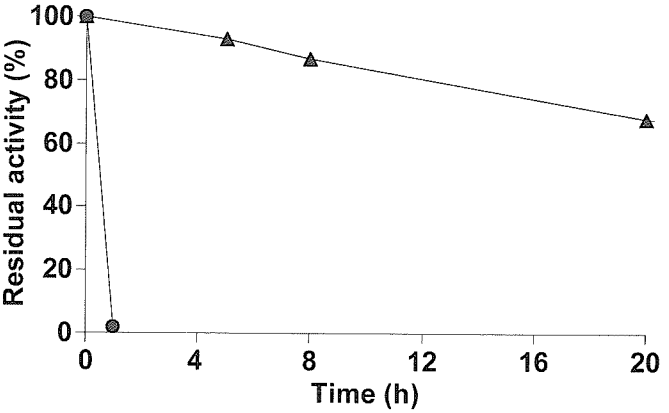

Fig. 4. Thermal stability of different GOX preparations. Triangle, immobilized on MANAE agarose activated with glutaraldehyde. Circle, soluble enzyme. Inactivation conditions were 56°C, pH 7.0, and 0.4 U/mL.

3. Filter the derivative. Add 1 g of derivative to 4 mL of 0.5% glutaraldehyde solution, pH 7.0. Leave for 1 h at 25°C.
4. Filter and wash the suspension with the excess 25 m*M* sodium phosphate, pH 7.0. Incubate for 18 h at 25°C.
5. Evaluate the covalent immobilization as described in **Subheading 3.5.**
6. This enzyme preparation is more thermostable than the soluble enzyme (*see* **Fig. 6**).

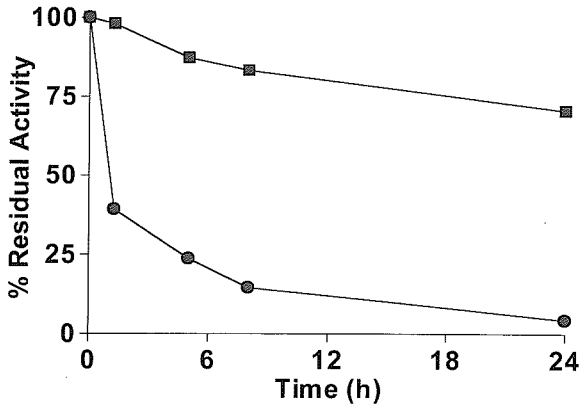

Fig. 5. Effect of cross-linking with glutaraldehyde on the thermal stability of DAAO. Circles, derivatives adsorbed onto Sepabeads EC-EA; squares, derivatives adsorbed onto Sepabeads EC-EA and then cross-linked with 0.5% glutaraldehyde solution. The inactivation course was carried out by incubating 0.8 U/mL DAAO in 10 m*M* potassium phosphate buffer, pH 7.0, at 50°C.

Fig. 6. Effect of cross-linking with glutaraldehyde on the thermal stability of the GA. Circles, derivatives adsorbed onto MANAE-agarose; squares, derivatives adsorbed onto MANAE agarose and then cross-linked with 0.5% glutaraldehyde solution. The inactivation course was carried out by incubating 10 U/mL of GA in 10 m*M* potassium phosphate buffer, pH 7.0, at 47°C.

4. Notes

1. The control of the pH is very important. If the pH is too high the glutaraldehyde will polymerize and the support will not react with the enzyme.
2. The suspension color is an indicator of the state of the glutaraldehyde. If the suspension is brownish in color the support cannot be used because glutaraldehyde reactivity is compromised.

3. The low-ionic strength
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4. The additional incubatio
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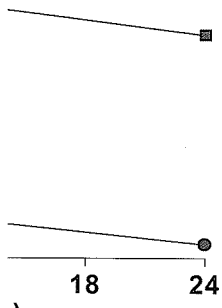

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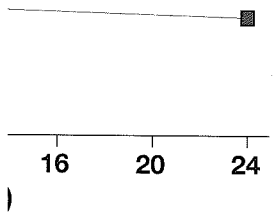

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3. The low-ionic strength of the immobilization buffer is necessary for the ionic adsorption between the protein and the support.
4. The additional incubation at 25°C is necessary to achieve a higher degree of cross-linking.
5. The low-ionic strength of the immobilization buffer permits the first ionic adsorption of the enzyme on the support. After this adsorption, the glutaraldehyde can covalently react with the primary amino groups of the protein, leaving the enzyme covalently attached to the support.
6. This experiment allows for the evaluation of the covalent attachment between the protein and the support.

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6

Practical Protocols Via Sol-Gel Techniq

Manfred T. Reetz

Summary

Lipases can be efficiently er and cheap sol-gel process in whi is hydrolyzed under basic condit propanol, polyvinyl alcohol, cy type of lipase immobilization biocatalysts concerns esterificati or supercritical carbon dioxide. I powders) of several orders of ma stability. The lipase immobilizat esters, enantioselectivity often b mercial forms of these lipases (j low price of sol-gel entrapment and the ready recyclability, this

Key Words: Lipases; sol-ge mal stability; kinetic resolution;

1. Introduction

A wide variety of enzymes many different transformation catalysts for synthetic organic can be used in nonaqueous me chemists to be performed that ment *(3)*. An example of signi 3.1.1.3) as catalysts in orga biocatalysts in synthetic organ

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Edited by: J. M.