**Covalent immobilization of lysozyme in silicone rubber modified by easy chemical grafting**

G. G. Flores-Rojas1,2,\*, F. López-Saucedo1, T. Isoshima2, E. Bucio1,

1Departamento de Química de Radiaciones y Radioquímica, Instituto de Ciencias Nucleares, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, México DF 04510, México.

2Nano Medical Engineering Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

\*Corresponding author: Tel.: +52 (55) 5622-4674

E-mail address: ggabofo@hotmail.com (G.G. Flores-Rojas)

 Corresponding author: Tel.: +52 (55) 5622-4674

E-mail address: ebucio@nucleares.unam.mx (E. Bucio)

***Supplementary information***

***S1. Experimental***

***S1.1. Materials***

Silicone rubber films (SR), with density from 1.1 to 1.5 g cm-3 and 1 mm in thickness, were purchased from Goodfellow (Huntingdon, UK). Ethylenediamine, tetrahydrofuran (THF), glutaraldehyde (50%) and azobisisobutyronitrile (AIBN), *Micrococcus lysodeikticus*, sodium cyanoborohydride, ethylene glycol dimethacrylate (EGDMA), and glycidyl methacrylate (GMA) were purchased from Sigma Aldrich (St. Louis, MO, USA), the monomers were distilled at reduced pressure previously to be used. Lysozyme was obtained from MP Biomedicals (Germany), and ethanol from J.T. Baker (Mexico). Coomassie Brilliant Blue G-250 (CBB) was purchased from Bio-Rad Laboratories, Inc., CA, USA.

***S1.2. Swelling study***

The swelling study was performed for pristine SR samples and SR samples with immobilized lysozyme. In the first case, the pristine SR samples were soaked in a monomers-toluene solution at different concentrations as swelling mediums, for a time of 4 h at room temperature. In the second case, the sample with immobilized lysozyme was immersed in bi-distilled water for 24 h at room temperature. After the swelling time, the films were removed from the solution and wiped with filter paper, and finally weighed. The swelling percentage was calculated as follows:

|  |  |
| --- | --- |
| $$S\left(\%\right)=100 [(W\_{s}-W\_{i})/W\_{i}]$$ | (Eq. S1) |

where *Wi* is the weight of the dry sample and *Ws* is the weight of the swollen sample, respectively.

***S1.3. Synthesis of SR-g-P(EGDMA-co-GMA), (SR-G)***

The SR (1×12 cm) were placed in an ampoule containing 22 mL of an EGDMA/GMA (1:1 mol) solution, varying the concentration from 10 to 100 vol.% of monomer in toluene with 132 mg of AIBN added as initiator. The ampoules were degassed by repeated freeze-thaw cycles (6 times per 10 min) and sealed at vacuum. Afterward, the ampoules were heated at 70 °C in water bath for a reaction time of 1 h. To extract the residual monomers and copolymer formed during grafting reaction, the samples were soaked in ethanol for 48 h (changing solvent), followed by drying under vacuum at room temperature to constant weight.

The grafting yield (*G*%) was calculated by the next equation:

|  |  |
| --- | --- |
| $$G\_{\%}=100 [(W\_{f}-W\_{i})/W]\_{i}$$ | (Eq. S2) |

where *Wf* and *Wi* are the weights of the initial and grafted SR, respectively.

***S1.4. Activation of SR-G and lysozyme immobilization***

The chemical activation of SR*-G* was made using 1x2.5 cm of sample film and placed in 5 mL of THF and 0.25 mL (3.74 mmol) ethylenediamine. The reaction was carried out at 75 °C under fixed stirring for 3 h. When the reaction was completed, the SR*-G* films modified with ethylenediamine (SR*-G-N*) were washed with a mixed solvent of distilled water and ethanol (70/30 vol.%) for 24 h.

Afterward, the SR-*G-N* samples were reacted with 650 mg (3.24 mmol) of glutaraldehyde in 5 mL THF at 30 °C for 24 h. When the films were removed from the reaction medium, they were washed with THF. Finally, the reduction of imine groups was performed in 5 mL THF and 50 mg (70 mmol) of sodium cyanoborohydride for 2 h at 30 ºC under constant stirring. The reaction was stopped using distilled water, and the obtained films (SR*-G-N-A*) were washed using distilled water.

Lysozyme immobilization was performed using a SR*-G-N-A* sample. This was placed in 2.5 mL of a lysozyme solution, with a concentration of 2 mg per mL of buffer solution pH=7, 0.01 M, at 5 °C for 72 h. Finally, the films with immobilized lysozyme (SR-*G-Lys*) were washed with NaCl solution 0.1 M and distilled water three times, 10 minutes each one, before carrying out the enzymatic activity test.

***S1.5. Non-specific test of lysozyme and quantification***

The non-specific test of immobilized lysozyme was carried out using Coomassie Brilliant Blue G-250 (CBB), which is a non-specific protein stainer. The samples of SR*-G-Lys* and SR*-G-N-A* were cut into small pieces of a few µm of thickness, and mm of wide and long to avoid the occlusion of CBB. The samples were washed firstly with ethanol for 1 h, then they were dried under vacuum for 12 h finally, it was added 1 mL of CBB after 1 h, and then the samples were washed with distilled water for 12 h.

Quantification of immobilized lysozyme was calculated by the remaining free lysozyme in the solution after the immobilization process as the equation 3 indicates. The free lysozyme in the solution was calculated by means of calibration curve [spectrophotometr](https://www.google.com.mx/search?q=spectrophotometry&spell=1&sa=X&ved=0ahUKEwjn9rOAgKLSAhXJgVQKHahjAgwQvwUIGCgA)ic at 280 nm, phosphate buffer pH 7, 0.01 M.

|  |  |
| --- | --- |
| $$Ly\_{m}=(Ly\_{fi}-Ly\_{ff})/A$$ | (Eq. S3) |

where *Lym* is the immobilized lysozyme, *Lyfi* and *Lyff* are the amounts of free lysozyme in the solution in the beginning and after the immobilization process of lysozyme, and *A* is the area in cm2 of the film.

***S1.6. Enzymatic activity test***

The enzymatic activity of SR*-G-Lys* was monitored by the decrease in absorbance at 450 nm, using 0.2 cm2 of SR*-G-Lys* and 4 mL of *M. lysodeikticus* suspension (0.6 mg mL-1, Ab*i* ≈ 0.6), at different temperatures, and pH values, 0.01 M.

The enzymatic activity was calculated using the equation Eq. 4 where *Abi* and *Abf* are the initial and the final absorbance measured in the system, *A* is the area in cm2, *t* is the time in minutes and 0.001 is the definition of unit enzymatic activity.

|  |  |
| --- | --- |
| $$Enzimatic activity \left(0.001min^{-1}cm^{-2}\right)= (Ab\_{i}-Ab\_{f})/(0.001At)$$ | (Eq. S4) |

***S1.7. Characterization of films***

FTIR-ATR spectra were recorded using a Perkin-Elmer Spectrum 100 (Perkin Elmer Cetus Instruments, Norwalk CT, USA) fitted with a Universal ATR sampling accessory (DiCompTM crystal, which is composed of a diamond ATR with a zinc selenide focusing element in direct contact with the diamond). Differential scanning calorimetry analyses were carried out in a DSC 2010 (TA Instruments, New Castle, DE) from 25 to 450 °C, under a nitrogen atmosphere at a flow rate of 60 mL min-1 and a heating rate of 10 °C min-1. Thermogravimetric analyses were performed using a TGA Q50 (TA Instruments, New Castle, DE) at a heating rate of 10 °C min-1 in the temperature interval from 25 to 800 °C under a nitrogen atmosphere. Kruss DSA 100 drop shape analyzer (Matthews NC, USA) was used to measure the water contact angle, which was recorded at 1 and 5 min after a bi-distilled water drop had been deposited on the dry films. The surface morphology was observed by AFM (MFP-3D, Asylum Research/Oxford Instruments PCI, CA. USA). The surface image was obtained in AC mode (tapping mode), using an NCH-W chip (Nanoworld Technologies AG, Switzerland). The mechanical properties of the samples were measured with a sample size of 5 x 0.5 cm using a tensile compression tester (model 1125, Instron Inc., MA, USA) at 23 °C and the analysis speed of 10 mm min-1.

***S2. Statistical analysis***

The statistical analysis was performed by analysis of variance (ANOVA) and using the comparison of Tukey method with a confidence of 95%

***S2.1.*** ANOVA of the grafting degree at different monomers concentration (vol.%).

|  |  |  |  |
| --- | --- | --- | --- |
| **Monomers concentration (vol.%)** | **N** | **Average of grafting degree (wt%)** | **Group (comparison Tukey method)** |
| **40** | 3 | 14.80 | A |  |  |  |  |  |
| **60** | 3 | 14.00 | A | B |  |  |  |  |
| **50** | 3 | 13.20 |  | B | C |  |  |  |
| **70** | 3 | 12.60 |  |  | C |  |  |  |
| **80** | 3 | 12.40 |  |  | C |  |  |  |
| **30** | 3 | 10.00 |  |  |  | D |  |  |
| **90** | 3 | 8.90 |  |  |  | D |  |  |
| **20** | 3 | 6.80 |  |  |  |  | E |  |
| **100** | 3 | 4.80 |  |  |  |  |  | F |
| **10** | 3 | 3.80 |  |  |  |  |  | F |

***S2.2.*** ANOVA of the contact angle study in the immobilization process of lysozyme.

|  |  |  |  |
| --- | --- | --- | --- |
| **Code** | **N** | **Average of contact angle (°)** | **Group (comparison Tukey method)** |
| **SR, 1 min** | 3 | 91.000 | A |  |  |  |
| **SR, 5 min** | 3 | 90.333 | A |  |  |  |
| **SR-G-N, 1 min** | 3 | 85.13 | A | B |  |  |
| **SR-G-N, 5 min** | 3 | 81.73 | A | B | C |  |
| **SR-G-Lys, 1 min** | 3 | 80.93 | A | B | C |  |
| **SR-G, 1 min** | 3 | 80.00 | A | B | C |  |
| **SR-G-Lys, 5 min** | 3 | 76.73 | A | B | C |  |
| **SR-G, 5 min** | 3 | 73.60 |  | B | C | D |
| **SR-G-N-A, 1 min** | 3 | 67.97 |  |  | C | D |
| **SR-G-N-A,  5 min** | 3 | 60.17 |  |  |  | D |

***S2.3.*** ANOVA of the enzymatic activity of the immobilized lysozyme in the series of SR-*G*.

|  |  |  |  |
| --- | --- | --- | --- |
| **Monomers concentration (vol.%)** | **N** | **Aaverage of enzymatic activity (0.001 min-1cm-2)** | **Group (comparison Tukey method)** |
| **40** | 3 | 11.000 | A |  |  |  |  |
| **50** | 3 | 10.000 |  | B |  |  |  |
| **60** | 3 | 9.903 |  | B |  |  |  |
| **70** | 3 | 7.403 |  |  | C |  |  |
| **100** | 3 | 4.100 |  |  |  | D |  |
| **80** | 3 | 3.800 |  |  |  | D |  |
| **90** | 3 | 3.6000 |  |  |  | D |  |
| **30** | 3 | 3.600 |  |  |  | D |  |
| **20** | 3 | 1.2000 |  |  |  |  | E |
| **10** | 3 | 0.7933 |  |  |  |  | E |

***S2.4***. ANOVA of the enzymatic activity study in different temperatures.

|  |  |  |  |
| --- | --- | --- | --- |
| **Temperature (°C)** | **N** | **Average of enzymatic activity (0.001 min-1cm-2)** | **Group (comparison Tukey method)** |
| **60** | 3 | 22.700 | A |  |  |  |
| **50** | 3 | 21.493 | A |  |  |  |
| **40** | 3 | 15.070 |  | B |  |  |
| **30** | 3 | 10.000 |  |  | C |  |
| **20** | 3 | 2.5000 |  |  |  | D |
| **70** | 3 | 1.4800 |  |  |  | D |

***S2.5.*** ANOVA of the enzymatic activity study in different pH values (0.01M).

|  |  |  |  |
| --- | --- | --- | --- |
| **pH** | **N** | **Average of enzymatic activity (0.001 min-1cm-2)** | **Group (comparison Tukey method)** |
| **8** | 3 | 14.330 | A |  |  |  |
| **6** | 3 | 9.000 |  | B |  |  |
| **10** | 3 | 8.500 |  | B |  |  |
| **7** | 3 | 8.000 |  | B | C |  |
| **9** | 3 | 6.790 |  |  | C | D |
| **5** | 3 | 6.270 |  |  |  | D |

***S2.6.*** ANOVA of the enzymatic activity study in different cycles.

|  |  |  |  |
| --- | --- | --- | --- |
| **Cycle** | **N** | **Average of enzymatic activity (0.001 min-1cm-2)** | **Group (comparison Tukey method)** |
| **1 °** | 3 | 10.500 | A |  |  |
| **5 °** | 3 | 5.700 |  | B |  |
| **3 °** | 3 | 5.640 |  | B |  |
| **4 °** | 3 | 4.833 |  | B | C |
| **2 °** | 3 | 4.230 |  |  | C |



**Fig. S1.** Infrared spectra of a) P(EGDMA-*co*-GMA), *ν*(CO) 1720 cm-1, *ν*(CC) 1638 cm-1, epoxide group *ν*(CO) 1091 and *ν*(CO) 994 cm-1 and b) Lysozyme, *ν*(CO) of amide I at 1646 cm-1, *ν*(CO) of carboxyl groups and amide II at 1517 cm-1.

****

**Fig. S2.** DSC thermograms of a) P(EGDMA-co-GMA), onset point 257.1°C, peak max. 290.9 °C, and b) lysozyme, onset point 100.3 °C, peak max. 125.8 °C, onset point2, 197.3 °C, peak max2. 202.2 °C.

****

**Fig. S3.** TGA thermograms of a) P(EGDMA-*co*-GMA), 242.0 °C at 10 wt. % loss and b) lysozyme, 220.8 °C at 10 wt. % loss.