Production of recombinant proteins by sol–gel immobilized
*Escherichia coli*

M.F. Desimone\(^a,1\), M.C. De Marzi\(^b,1\), G.J. Copello\(^a\), M.M. Fernández\(^b\),
F.L. Pieckenstain\(^c\), E.L. Malchiodi\(^b\), L.E. Diaz\(^a,\ast\)

\(^a\) Cátedra de Química Analítica Instrumental, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 Piso 3° (1113) Ciudad de Buenos Aires, Argentina
\(^b\) Cátedra de Inmunología, IDEHU-CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 Piso 3° (1113) Ciudad de Buenos Aires, Argentina
\(^c\) Laboratorio de Fijación Simbiótica de Nitrógeno, Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada, Campus Fuentenueva s/n (18071) Granada, Spain

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Abstract

The aim of this work was to study the functionality and quantitative level of recombinant protein produced from bacteria immobilized and preserved in sol–gel matrices. Matrices prepared from two precursors, silicon dioxide and tetraethoxysilane, were studied. In previous works we analyzed the number of viable cells and level of recombinant protein production from cultures started with immobilized *Escherichia coli*, stored in sealed tubes at 4 and 20°C. We observed that the amount of bacteria in silicon dioxide derived matrix conserved in the same order of magnitude as before immobilization, during 2 months, but those in an alkoxide derived matrix decrease until no viable cells were detected at both 4 and 20°C after 42 days. In this work, immobilized bacteria were used as culture starter to produce recombinant proteins with a yield comparable with glycerol stocks. T-cell proliferation and gel filtration assays suggest that SAgs produced from cultures started with sol–gel immobilized bacteria retain their biological activity. Affinity assays using a resonant biosensor showed that Streptococcal Superantigen (SSA) has affinity for human Vβ5.2 produced from sol–gel immobilized bacteria with *K_* \(_D\) = 7.5 \(\mu\)M. These results contribute to the development of methods for microbial cells preservation under field conditions.

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1. Introduction

The sol–gel matrix is highly porous, possesses mechanical strength, chemical and thermal stability and provides a hydrophilic polymer that can be used as a support for immobilization processes. Different applications of sol–gel technology have been studied [1]. The mild conditions associated with sol–gel chemistry allows the successful immobilization of a broad range of enzymes but fewer reports involving living cells have been made [2,3]. The synthesis of solid inorganic materials from alkoxide and aqueous routes are alternatives areas being developed to improve the viability of encapsulated microorganisms [4]. The use of additives such as glycerol and polyethylene glycol, biocompatible pH, and the elimination of cytotoxicity of the silica sol – basically due to alcohol by products of alkoxide hydrolysis during the immobilization processes – improve the viability after immobilization [5,6].

With the purpose of evaluating microbial survival and recombinant protein production in inorganic matrices, we have previously studied the use of silicon oxide matrices for the immobilization and preservation of three *Escherichia coli* transformants. One was transformed with the chimerical TCR \(\beta\) chain (hVβ5.2mCβ1), which is expressed as inclusion bodies in the cytoplasm. The two others were transformed with the bacterial SAgs Streptococcal Superantigen (SSA) and Staphylococcal Enterotoxin G (SEG), expressed as soluble protein in the periplasm [7]. We proposed an alternative method...
for long-term preservation without the need for low temperature freezers. This will contribute to microbial cells preservation under field conditions and transportation, especially in tropical countries.

Herein, we study the functionality and the production of recombinant proteins obtained from cultures started with sol–gel immobilized E. coli stored for 2 months at 4 and 20 ºC. SSA and SEG produced from sol–gel immobilized bacteria were used to study T-cell proliferation capacity with human peripheral blood mononuclear cells (PBMCs). The binding of SSA to soluble hVß5.2mCß1 produced from sol–gel immobilized bacteria was measured in real time by a resonant mirror optical biosensor method. We use immobilized E. coli, as a model microorganism that remains with their metabolic activity intact to produce the recombinant proteins under study preserving their biological activity.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Transformed E. coli BL21 (DE3) from glycerol stocks (hVß5.2mCß1, SSA and SEG, cloned in a kanamycin-resistant expression vector pET 26b+) were plated on LB-agar with 50 µg/ml kanamycin and incubated at 37 ºC overnight. Some colonies from different transformants were cultured in LB medium with kanamicyn up to OD (600 nm) 0.800, centrifuged and resuspended in LB medium with 100 mM sodium phosphate buffer, pH 8.0 and glycerol (20%). The colony-forming units per ml of this suspension were determined by the plate count technique and then used in immobilization and preservation studies.

2.2. Immobilization

The sol was prepared by sonicating a mixture of 1 ml TEOS, 0.2 ml water and 0.06 ml of 0.04 M HCl for 30 min at 20 ºC. After addition of 2 ml of water, the ethanol excess was eliminated under N2. Bacterial suspension of 1 × 109 CFU/ml was mixed with an equal volume of the sol solution. The solution was allowed to stand 2 min for gelation. Alternatively, the matrix was prepared by acidification to pH 8.0 of a solution of sodium silicate (1.1 g SiO2, 4 ml of 2 M NaOH). Bacterial suspension of 1 × 109 CFU/ml was mixed with an equal volume of a five-fold dilution of the sodium silicate solution. Gelation occurred immediately.

2.3. Protein production and purification

LB-agar plate culture with 50 µg/ml kanamycin was grown overnight (ON) at 37 ºC from E. coli BL21 (DE3) glycerol stock. Preculture (20 ml) was grown at 30 ºC from one single colony. Alternatively one sol–gel matrix coming from SiO2 derived matrix (10 or 60 days post-immobilization) or in TEOS derived matrix (10 days post-immobilization), stored at 4 or 20 ºC, were added to 1000 ml LB culture medium. Bacteria were grown until turbidity OD (600 nm) reached 0.800 (4 h) and then induced with isopropyl-ß-D-thiogalactopyranoside (IPTG) and cultured for 5 h.[8]

The periplasmic fraction, which contained most of the SAg, was obtained by osmotic shock as described previously[9] and further purified by Ni2+-NTA affinity chromatography as described by the manufacturer (Qiagen, Valencia). Further purification of SAgS was performed with a size exclusion Superdex 75 column and finally in a Mono-S cation exchange column (Amersham Biosciences, Piscataway, N.J.)[10]. TCR expression, refolding and purification were performed as described by De Marzi et al.[10]. Protein yield was determined measuring the absorbance at 280 nm.

2.4. T-cell proliferation assay

Heparinized blood was obtained from healthy blood donors, previously tested for antibodies against SSA and SEG by ELISA with negative results, with the understanding and written consent of each subject. Twenty milliliters of diluted blood (with RPMI 1640) was slowly added to 10 ml of Ficoll-PaqueTM (Amersham Biosciences) and centrifuged at 400 g for 20 min. The PBMCs were washed with 20 ml of RPMI and centrifuged for 10 min at 200 g. The pellet was resuspended in 5 ml of RPMI with 10% of human serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM pyruvate. The PBMC population was counted with Trypan Blue in a Newbauer camera.

Purified cells (105 per well) were cultured in flat-bottom 96-well plates in the presence of varying dilutions of staphylococcal enterotoxin C3 (SEC3) as control, SSA and SEG from immobilized and non-immobilized bacteria in 100 µl of complete culture medium. Phytohaemagglutinin (1 µg/ml) was used as positive control. After 48 h incubation at 37 ºC in 5% (v/v) CO2, 1 ml per well of [3H]-thymidine was added for the next 18 h and then harvested onto glass fiber filters. Incorporation of radioactivity was measured using a Liquid Scintillation Analyzer 1600 TR (Packard, Canberra, Australia). All measurements were made by triplicate.

2.5. Gel filtration assay

The interaction between SEG, produced from sol–gel immobilized bacteria, and mouse TCR ß chain mVß2.8 was detected by gel filtration as described previously by Petersson et al.[11] using an AKTA purifier system (Amersham Pharmacia Biotech, Upsala, Sweden). Briefly, SEG and mVß2.8 were incubated at concentrations of 5 µM for 2 h at 37 ºC. The protein mixture was applied to a gel filtration S200 column equilibrated with PBS. The column was previously calibrated with aldolase, BSA, OVA, chymotrypsinogen A and ribonuclease A as molecular weight standards; the void volume (Vv) was determined using Blue dextran 2000 (Amersham Biosciences, England).

2.6. Surface plasmon resonance SPR

The interaction of soluble hVß5.2mCß1 chain with SSA was monitored in a resonance mirror with an IAsys instrument (Labsystem, Cambridge, UK) biosensor, which allows determination of real time interactions between two molecules. SSA (100 µg/ml) was dialyzed against 10 mM sodium acetate, pH 5.5, and coupled to the carboxymethyl-dextran cuvettes (Labsystems) using the Amine Coupling Kit as described by the manufacturer. The activation and immobilization periods were set between 5 and 7 min to couple the desired amount of proteins yielding between 400 and 600 arc seconds. Vß5.2, produced from sol–gel immobilized bacteria, was dialyzed against PBS containing 0.05% (v/v) Tween 20. Twofold dilutions were made in the same buffer (40, 20, 10, 5 and 2.5 µM). All binding experiments were performed at 25 ºC. Dissociation was carried out in PBS Tween 20. Pulses of 10 mM HCl were used to regenerate the surface. All the experiments were repeated at least three times. Dissociation constants (Kd) were determined under equilibrium binding conditions using Scatchard plots after correction for non-specific binding, in which the proteins were passed over blocked, empty cuvettes.

3. Results

Protein production studied 10 days post-immobilization, found similar yields in the amount of recombinant proteins produced from bacteria immobilized in different matrices and non-immobilized ones. No significant differences were found when we compared recombinant protein yield coming from cultures started with sol–gel immobilized bacteria stored at different temperatures (4 and 20 ºC). This is concordant with previous viability studies. Since beyond day 42 no viable cells were found in the TEOS derived matrix, we only analyzed recombinant protein production on day 60 in cultures started from
SiO\textsubscript{2} derived matrix stored at 4 or 20 °C. In all cases protein yield was \(\sim 15\) mg/l for SAgs and \(\sim 2\) mg/l for TCR chain.

The ability of SSA and SEG, produced by the different sol–gel immobilized bacteria stored at 4 or 20 °C after 10 or 60 days post-immobilization, to stimulate human T-cells was analyzed. Different SSA and SEG preparations yielded dose-dependent T-cell proliferation, analyzed by \([3\text{H}]\)-thymidine incorporation. This result showed that these SAgs were fully active, as those produced by non-immobilized bacteria. Fig. 1 shows a representative assay for SAgs produced in cultures started with bacteria immobilized in SiO\textsubscript{2} derived matrix stored at 20 °C for 60 days.

The ability of SEG to interact with mV/\textsubscript{H9252}8.2 chain was previously reported by Fernández et al. [12]. Size exclusion chromatography was performed to confirm that SEG produced in cultures started with bacteria immobilized in sol–gel matrix indeed binds V/\textsubscript{H9252}8.2 chain. On a previously calibrated Superdex 200 column, we loaded highly purified SEG, mV/\textsubscript{H9252}8.2 chain or a mixture containing equimolar quantities of both proteins incubated for 2 h at 37 °C. Fig. 2 shows a peak of 45 kDa that corresponds to SEG–mV/\textsubscript{H9252}8.2 complex. Another two peaks of 30 and 15 kDa come out of the column later, corresponding to SEG and mV/\textsubscript{H9252}8.2 monomers, respectively. The peaks were analyzed by 20% SDS-PAGE to confirm the presence of the proteins as complex or monomers.

Equilibrium parameters for SSA binding to TCR chain, the last one produced in cultures started with bacteria immobilized in SiO\textsubscript{2} derived matrix, were determined in a resonance mirror using an IAsys instrument biosensor. As shown in Fig. 3, TCR V/\textsubscript{H9252}5.2 concentrations dependent binding to SSA was observed. Therefore, affinities (\(K_D\)) were determined under equilibrium binding conditions, in which we took report points for Scatchard analysis 4 min after injection. The \(K_D\) value obtained was 7.5 \(\mu\)M which is similar to that obtained for protein produced by non-immobilized bacteria [10]. Similar results were obtained using TCR V/\textsubscript{H9252}5.2 chain produced in cultures started with SiO\textsubscript{2} and TEOS immobilized bacteria for both temperatures and storage times assayed.
4. Discussion

Previous results [7] showed that the immobilization in a matrix obtained from SiO₂ precursors was effective for the preservation of transformed E. coli with high viability rates and without contamination. This SiO₂ derived matrix with immobilized bacteria generates a suitable microenvironment, which could be efficiently stored either at 4 or 20 °C in sealed tubes without contamination for at least 60 days. Although wet TEOS derived matrices had better mechanical properties, it was difficult to completely eliminate the ethanol generated during the polymerization reaction reducing viability rates; this drawback was absent in SiO₂ derived matrices.

Cells must remain with their metabolic activity intact to produce the recombinant proteins under study. Protein production from bacteria immobilized in different sol–gel derived matrices, at both temperatures and storage time, yield the same amount as those obtained from glycerol stock for the three molecules under study. In addition, recombinant proteins are recognized by their specific antibodies, confirming the preservation of the interaction sites and, thus, the preservation of the protein structures [7].

Since immobilization or adsorption on inert matrices may cause alterations in the metabolic behavior of cells, as described in several studies revealing altered metabolic regulation, increased ethanol tolerance [13], and modifications in membrane fatty acids composition [14], more information is necessary on the physiology of immobilized cells, specially for those that produce specific products in which genetic stability and intact metabolic pathways are relevant.

In this work, immobilized bacteria were able to produce fully biological active proteins as it was confirmed by human T-cell proliferation and gel filtration assays with SAg s and by affinity studies using biosensor technology.

TCR β chain functionality analyzed in a resonance mirror biosensor, demonstrated that the protein produced from sol–gel immobilized bacteria was fully active, since the $K_D$ obtained for SSA–Vß5.2 interaction is in the same order of magnitude than those produced from glycerol stocks [10]. The fact that Vß5.2 is produced as inclusion bodies in the cytoplasm and renaturation was performed in vitro, suggests that the recombinant protein synthesis was successful. In order to further analyze the recombinant protein folding capability of the bacteria, we immobilized bacteria producer of SAg s. These molecules were produced as soluble periplasm proteins, requiring to be folded by the bacterium. Proliferation studies demonstrated that SAg s obtained from immobilized bacteria were fully biological active. These means that they were able to interact with TCRs and MHC II molecules presented in T cells and antigen presenting cells, respectively, showing that SAg s interaction sites were preserved without modifications. In addition, we demonstrated that SEG produced by immobilized bacteria is able to bind mVß8.2 since it is possible to isolate the complex SEG–mVß8.2 by size exclusion chromatography.

This work contributes to the study of new matrices with the capability of conserving different bacteria strains, for use in biochemical or biotechnological processes.

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