Production of monoclonal antibodies from hybridoma cells
immobilized in 3D sol-gel silica matrices

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Abstract
The immobilization of mammalian cells in suitable matrices that can retain their viability and capability to produce certain metabolites has gained attention in recent years. In this work, hybridoma cells were immobilized in sol-gel silica matrices for \textit{in vitro} production of monoclonal antibodies. For that purpose, different matrices were evaluated in terms of cell viability, antibody diffusion to surrounding media and physicochemical properties of the polymeric material. Tetrakis (2-ethoxyethyl) orthosilicate (THEOS) matrices were found to be the best option for hybridoma immobilization. The concentrations of the silica precursor as well as the number of immobilized cells were also optimized. Three hundred mM of THEOS precursor and $5 \times 10^5$ hybridoma cells appear to be the most suitable alternative. Hybridoma cells immobilized in THEOS matrices were able to produce monoclonal antibodies to the same extent as free cells, thus introducing the possibility of using them in the design of bioreactors for large-scale production.

Keywords: sol-gel, encapsulation, hybridoma, immobilization, monoclonal antibodies.

INTRODUCTION
The immobilization of mammalian cells in suitable matrices that can retain their viability and capability to produce certain metabolites has gained attention in recent years due to the possibility of using these materials with therapeutic purposes in tissue transplantation, development of bioartificial organs or large-scale production of metabolites in bioreactors \cite{1}. Numerous mammalian cell encapsulation techniques, which are generally classified as micro or macro encapsulation, have been developed. Mammalian cells are widely used to produce recombinant glycoproteins such as hormones, enzymes, and antibodies for human therapy. Among them, monoclonal antibodies (MAbs) produced by hybridoma cells, are in the group of products derived from large-scale mammalian cell cultures that have shown a rising demand in many areas of research, diagnostics and therapy. The high amounts of MAbs needed, especially for therapeutic applications, make their production a key factor with a growing interest in the use of immobilized hybridoma cells. The advantage of using these systems over free cells includes protection from shear damage and the possibility of employing immobilized cells in continuous operations, which maintain high cell densities and result in higher MAb production rates. Moreover, as an additional advantage, immobilized hybridoma lines require less amounts of fetal calf serum in the preparation of hybridoma media. Sol-gel technology consists in the polymerization of inorganic silica precursors taking place in mild, biocompatible conditions and has proved to be a suitable tool for the immobilization of biomolecules, microorganisms, parasites, etc \cite{2}. Furthermore, these inorganic matrices have also been successfully used for the entrapment of animal cells \cite{3}. Alternatively, the development of hybrids and composites has been recognized as a promising strategy to fulfill the complex requirements of scaffolds for cell-based tissue engineering applications. In this work we report the immobilization of hybridoma cells in sol-gel derived silica matrices which maintain the monoclonal antibody production capability. In addition, the influence of different sol-gel precursors for the entrapment of the cells, their viability over time and the production and release of MAbs were carefully analyzed.
MATERIALS AND METHODS
Immobilization in 3D sol-gel silica matrices
Cells were immobilized by using three different silica precursors: sodium silicate, TEOS and THEOS. For sodium silicate, 1 g of sodium silicate was mixed with 6 ml water and then incubated at 80 °C until a complete colloidal suspension was obtained. Each sol (colloidal suspension) was allowed to reach room temperature and acidified to pH = 6.5 with 0.75 M citric acid. Afterwards, a cell suspension in supplemented RPMI was mixed with an equal volume of the above described colloidal suspension. When TEOS was used, the sol solution was prepared by sonicating a mixture of 1 ml TEOS, 0.2 ml water and 0.06 ml 0.05 M HCl for 30 min at 20 °C. After addition of 2 ml water ethanol excess, product of tetraethylorthosilicate hydrolysis was eliminated under N₂. A cell suspension was mixed with an equal volume of the sol solution. The solutions were left for 2 min until gelation. Finally, when THEOS was used as the sol-gel precursor, the cell suspension was mixed with an equal volume of the precursor and left until gelation occurred. Sol-gel silica matrices with medium but without cells were employed as negative controls and nonimmobilized cells as positive controls. Cellular viability of entrapped cells was determined by the tetrazolium assay (MTT).

Antibody production and release from silica matrices
Purified rabbit polyclonal antibodies anti-mouse IgG were immobilized in sodium silicate or THEOS matrices with or without the addition of bovine serum albumin (BSA) or bovine fetal serum (BFS). The protein solutions in PBS were mixed with an equal volume of the colloidal suspension as described above. Each flask containing the entrapped antibodies (1 mg/ml) in THEOS and sodium silicate matrices was incubated with PBS at 37 °C for 48 h. Periodically, 1 ml aliquots were taken to determine antibody released from the matrices by SDS-PAGE and ELISA with mice IgG immobilized plates. Each 75 cm² flask containing free and entrapped hybridoma cells in THEOS matrices were incubated with RPMI media at 37 °C in a humidified 5% CO₂ air atmosphere for 30 days. Periodically, 1 ml aliquots were taken to determine antibody production by ELISA.

RESULTS AND DISCUSSION
Evaluation of cell viability in various 3D sol-gel silica matrices
Metabolic activity (24 h) was 80% of the initial cell number in THEOS matrices. At the same time, the cell viability in TEOS and silicate matrices was near 40% of the initial cell number. On day 6, viability was almost the same in sodium silicate matrices or higher in THEOS matrices than when initially used in the immobilization process. On the contrary, TEOS immobilized cells followed a decreasing kinetic showing no cell alive by day 8. It is worth mentioning that, after the 24 h reduction of immobilized cells, the kinetic growth for free, THEOS and sodium silicate immobilized cells was similar, showing the highest cell viability on day 6. Therefore, polyol-modified silanes are excellent candidates for cell immobilization, especially due to the biocompatibility of diol or polyol released upon hydrolysis. Furthermore, THEOS derived matrices succeed in maintaining cell viability even in higher levels than aqueous sol-gel precursors denoting the biocompatibility of both immobilization processes (data not shown).

Antibody release
The immobilized polyclonal antibodies showed a strong adsorption to matrices obtained with sodium silicate. On the contrary, the antibodies released from THEOS-derived matrices during the first 24 h was significantly higher as shown in Fig. 1. Indeed, the presence of polyols produced during the hydrolysis of THEOS, can reduce the adsorption of proteins onto silica, presumably due to the preferential coating of silica with organic additives. In addition, to demonstrate that the biological effect of the immobilized antibodies was conserved after immobilization in the THEOS matrix, we analyzed the kinetic release of rabbit polyclonal antibodies by ELISA. Fig. 2 shows that the rabbit antibodies can be detected immediately after gel formation and the antibodies in the supernatant reach equilibrium with a constant value of 35% after 48 h, without replacing the media.
Cell density and “Si” concentration

The viability of hybridoma cells immobilized in four cell densities within sol-gel silica matrices, obtained with four THEOS concentrations, was analyzed by MTT. When the number of cells before immobilization was 1.5 x 10⁶ or 1 x 10⁶, the cell metabolic activity did not recover during the 15-day period of culture, and remained at 30% or 40% of the initial activity, respectively (Fig. 3a and 3b). At 5 x 10⁵ cells, after the first reduction, the activity remained near the same level than 24 h (Fig. 3c). Surprisingly, when 2.5 x 10⁵ cells were encapsulated, a fast increase of the metabolic activity was detected since the first 24 h until day 15 (Fig. 3d). Similarly, the concentration of the sol-gel precursor strongly affected the metabolic activity of the cells with an inverse correlation. Thus, the highest cell metabolic activity at 24 h after immobilization was observed at the lowest precursor concentrations (150 mM) for all the cell numbers assayed (Fig. 3). This phenomenon would be related to the fact that softener gels resemble the usual culture condition of the cell line, thus favoring cell proliferation. However, silica gels in low concentrations are mechanically unstable and break easily, which are unfavorable properties for a 3D culture system intended for production of MAbs with immobilized cells. In fact, cells are better adapted to lowly concentrated gels for all the densities of immobilized cells. In view of these results, 300 mM appear to be a compromise concentration for the silica precursor in order to have an adequate matrix that allows the survival of most of the cells in the gel forming process.

**Fig. 1.** Release of rabbit polyclonal antibodies were analyzed by SDS-PAGE. 1. Negative control for silicate matrix (no antibody added); 2. Silicate with BFS; 3. Silicate with albumin; 4. Silicate matrix; 5. THEOS matrix; 6. THEOS with BFS; 7. THEOS with albumin; 8. THEOS matrix; 9. Negative control for THEOS; and 10. Positive control.

**Fig. 2.** Release of immobilized rabbit polyclonal antibodies. THEOS matrices (●) and THEOS matrices supplemented with BFS (▲) or albumin (■) were used to immobilize rabbit polyclonal antibodies against mice IgG. The antibodies detected in the supernatant by ELISA are expressed as percentage of the immobilized antibodies in function of time. Each bar represents the group mean ± SD. The results are representative of three independent experiments.

**Fig. 3.** MTT assay for hybridoma cells immobilized in THEOS-derived matrices. Different concentrations of the sol-gel precursor (150 mM ●; 300 mM ■; 450 mM ▲ and 600 mM ▼) were used to immobilize the following number of hybridoma cells: a) 1.5 x 10⁶ b) 1 x 10⁶ c) 5 x 10⁵ and d) 2.5 x 10⁵. A 100% value has been assigned to MTT activity of each initial cell suspension. Each bar represents the group mean ± SD. The results are representative of three independent experiments.
Monoclonal antibody production by hybridoma cells within 3D sol-gel silica matrices

Immobilized cells were able to produce monoclonal antibodies in similar amounts to those in free hybridoma cells, when the initial number of cells added to culture media was $1 \times 10^6$ (Fig. 4). However, when the number of immobilized cells was lower ($5 \times 10^5$), it was observed that higher levels of MAbs were recovered from the culture media in shorter times. This fact is in concordance with the results presented for hybridoma metabolic activity determined by MTT.

CONCLUSIONS

THEOS matrices in this work demonstrated to be an alternative support for the successful immobilization of hybridomas in 3D matrices and therefore for the in vitro production of MAbs with biological activity as demonstrated by ELISA. Correlations between hybridoma cell viability and culture material composition and properties have been established. Thus, 300 mM of THEOS precursor and $5 \times 10^5$, as initial number of cells, would be the most suitable alternative. Moreover, immobilized cells are retained in the polymeric matrix simplifying the purification of the desire product which is easily released to the media. Indeed, the risk of contamination of immobilized cells is lower than in the case of cell suspensions. Accordingly, a new material has been introduced for the immobilization of cells showing better results in comparison with other matrices obtained through the sol-gel process such as TEOS or silicate-derived materials.

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