In Vitro Characterization and In Vivo Functionality of Erythropoietin-Secreting Cells Immobilized in Alginate−Poly-L-Lysine−Alginate Microcapsules

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The in vitro and in vivo characterization of cell-loaded immobilization devices is an important challenge in cell encapsulation technology for the long-term efficacy of this approach. In the present paper, alginate−poly-L-lysine−alginate (APA) microcapsules containing erythropoietin (Epo)-secreting C_2C_{12} myoblasts have been elaborated, characterized, and tested both in vitro and in vivo. High mechanical and chemical resistance of the elaborated microcapsules was observed. Moreover, the in vitro cultured encapsulated cells released 81.9 ± 8.2 mIU/mL/24 h (by 100 cell-loaded microcapsules) by day 7, reaching the highest peak at day 21 (161.7 ± 0.9 mIU/mL/24 h). High and constant hematocrit levels were maintained over 120 days after a single subcutaneous administration of microcapsules and lacking immunosuppressive protocols. No major host reaction was observed. On the basis of the results obtained in our study, cell encapsulation technology might be considered a suitable therapeutic strategy for the long-term delivery of biologically active products, such as Epo.

I. Introduction

In the few last decades, researchers involved in the development of pharmaceuticals have understood that drug delivery is a fundamental part of drug development. This issue is particularly relevant considering that over 95% of all new potential therapeutics have poor pharmacokinetics and biopharmaceutical properties.[1] In addition to reducing the frequency of drug administration and thus improve patient comfort, novel drug delivery systems would offer protection and improve the pharmacokinetics of easily degradable peptides and proteins, which often have short half-lives in vivo.[2]

Although standard drug therapy is usually effective in treating the symptoms of a disorder, a patient may be required to take the drugs for an extended time, and there may be serious or unpleasant side effects. However, a patient may be cured with few negative consequences if the treatment can be targeted directly at the specific cause of the disease (the gene defect) or if that cause can be neutralized or reversed. Therefore, gene therapy provides an attractive alternative to drug therapy as it seeks to provide treatment strategies that will be more complete and less toxic to the patient. Furthermore, gene therapy may provide a way of treating diseases that cannot be managed by standard therapies. As an example, the search for alternative therapies to the continuous injections that recombinant human erythropoietin (rHuEpo) treatments require is at its peak.

One of the emerging technologies that has gained the attention of the scientific community is cell encapsulation. Cell encapsulation results in the immobilization of cells in biocompatible as well as chemically and mechanically stable devices that deliver "de novo" produced therapeutic products in a sustained and controlled manner. Besides, the protection of the inner cell content from both mechanical stress and the host's immune response is ensured. This could be an advantage, as chronic administration of immunosuppressants could be avoided, improving the quality of life in patients again.

An important issue to be considered is the adequate nutrient and oxygen supply to encapsulated cells. In vascularized tissue, the maximum distance for an effective diffusion of oxygen and nutrients from capillaries to cells is 200 μm. Thus, it is recommended to keep the distance between cells and the vasculature to a minimum to provide cells with the necessary nutrients and oxygen. Moreover, a convenient adaptation of the scaffold to the selected cell source is required. The microenvironment of the device should mimic the extracellular matrix or the culturing conditions in which the enclosed cells grow.

This strategy has provided a wide range of promising therapeutic treatments for central nervous system diseases,[3−7] diabetes,[8−12] hemophilia,[13−14] and anemia[15] among others. This technology offers a safe and manufacturable method for the local and systemic delivery of therapeutic molecules from the enclosed cells. It can be considered as a "living drug delivery system" where the transplanted cells provide an unlimited drug source. As long as the cells are viable and functional, they are able to release the desired products in a more physiological manner. The microcapsule's membrane can serve as an immunoisolation barrier to keep the host's immune system away from the living cells, but at the same time it allows nutrients, oxygen, waste, and cell products to pass through without much difficulty.

Scientists are now taking steps to properly resolve some of the main challenges of this field[16−18] including the selection of clinical-grade biopolymers,[19] the development of a standardized, repeatable, and reproducible technology,[20−21] the control of permeability, mechanical stability, and durability of the microcapsules,[22] and, last but not least, the suitable in vivo evaluation of the microcapsules.

Recently, as a proof of principle, we have studied cell encapsulation technology by implanting encapsulated Epo-secreting cells in the peritoneum and subcutaneous tissue of syngeneic and allogeneic mice.[23] Epo was selected as a model drug because of its emerging therapeutic effects and due to the

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ease of monitoring its expression and bioactivity in vivo by following the hematocrit level. In addition, due to its short half-life, we suggest that cell encapsulation technology could avoid the tiresome repeated Epo injections currently practiced. Despite our initial positive results, further characterization and evaluation of the microcapsules and longer in vivo evaluation periods were suggested to properly evaluate the safety and long-term functionality of this approach.

In the present paper, a complete morphological and mechanical evaluation of microcapsules containing Epo-secreting C_2C_{12} myoblasts has been carried out. Furthermore, the in vitro characterization and the in vivo functionality and biocompatibility of the encapsulated cells during 4 months have been studied and discussed.

II. Experimental Procedures

Cell Culture. Murine C_2C_{12} myoblasts derived from the skeletal leg muscle of an adult C3H mouse and genetically engineered to secrete murine Epo (mEpo) were kindly provided by the Institute des Neurosciences (Ecole Polytechnique Federale of Lausanne (EPFL), Lausanne, Switzerland). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine to a final concentration of 2 mM, 4.5 g/L glucose, and 1% antibiotic/antimycotic solution. Cultures were plated in T-flasks, maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere standard incubator, and were passaged every 2−3 days. All reagents were purchased from Gibco BRL (Invitrogen S.A., Spain).

Before cell encapsulation, Epo secretion from 10^6 cells/mL/24 h was determined using a sandwich enzyme-linked immunoabsorbent assay (ELISA) kit for human Epo (R&D Systems, Minneapolis, MN). Cross-reaction of the kit allowed detection of mEpo in culture supernatants.

Cell Encapsulation. C_2C_{12} myoblasts genetically engineered to release Epo were immobilized into alginate−poly-L-lysine−alginate (APA) microcapsules using an electrostatic droplet generator with brief modifications of the procedure designed by Lim and Sun.[24] Low viscosity and high guluronic (LVG) alginate was purchased from FMC Biopolymer (Norway), and poly-L-lysine (PLL; hydrobromide M_w, 15 000−30 000 Da) was obtained from Sigma (St. Louis, MO). Briefly, cells were suspended in 1.5% (w/v) LVG-alginate sterile solution, obtaining a cell density of 2×10^6 cells/mL of alginate. This suspension was extruded through a 0.35 mm needle using a 10 mL sterile syringe with a peristaltic pump. The resulting alginate beads were maintained in agitation for 10 min in a $CaCl_2$ solution (55 mM) for complete ionic gelation and were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a coating with 0.1% alginate for another 5 min. Microcapsules were prepared at room temperature, under aseptic conditions, and were cultured in complete medium. The diameters and overall morphology were characterized using inverted optical microscopy (Nikon TSM) and confocal microscopy (Olympus FluoView FV500). Fluorescence images were obtained applying a viability/cytotoxicity test for mammalian cells purchased from Invitrogen.

Mechanical Stability Studies: Compression and Osmotic Resistance Tests. The compression resistance of microcapsules was determined as the main force (*g*) needed to generate 70% uniaxial compression of a sample of microcapsules using a Texture Analyzer (TA-XT21, Stable Microsystems, Surrey, U. K.). The force exerted by the probe on the microcapsule was recorded as a function of the compression distance leading to a force versus strain relation. Thirty microcapsules per batch were analyzed to obtain statistically relevant data.

The swelling behavior of the microcapsules was determined after 1% citrate solution (w/v) treatment. In short, 100 μL of microcapsule suspension (50−100 microcapsules) was mixed with 900 μL of phosphate-buffered saline (PBS) and placed in a 24-well cell culture cluster. Four wells were used for each group. The cell cluster was placed in a shaker at 500 rpm and 37 °C for 1 h. Afterward, supernatants were eliminated, and 800 μL of a sodium citrate solution was added. The cluster containing the microcapsules was maintained at static conditions at 37 °C for 24 h. On the following day, the diameters of 20 microcapsules of each group were measured. The washing and shaking step with PBS and the static condition were repeated during the following days until a 6-day period was completed.

mEpo Production and Metabolic Cell Activity. The cellular activity and Epo secretion of the entrapped cells were evaluated in vitro for 21 days. The viable cell number per microcapsule was determined by the tetrazolium assay (MTT) (Sigma, St. Louis, MO). Briefly, 25 μL of a 5 mg/mL solution of MTT in PBS was added to a known number of microcapsules (around 40) placed in a 96-well cell culture cluster and incubated at 37 °C for 4 h. Afterward, the MTT solution was removed by vacuum aspiration, and 100 μL dimethylsulfoxide was added. The resulting purple solution was read 5 min later on a microplate reader (Multiskan EX Labsystems) at 560 nm with 690 nm as the reference wavelength. Results are expressed as mean ± standard deviation.

Conditioned media samples (cell supernatants) were assayed using the Quantikine IVD Epo ELISA kit purchased from R&D Systems. Standards and samples were run in duplicate according to the procedure specified in the kit. The detection limit of this assay was 2.5 mIU/mL. The mEpo secretion of around 200 cell-loaded microcapsules was measured in conditioned medium for an 8 h release period to calculate the C_2C_{12}-mEpo-microencapsulated cells daily secretion rate. Results are expressed as mean ± standard deviation.

Microcapsule Implantation. Adult female Balb/c mice (Harlan Interfauna, Spain) were used as allogeneic recipients. Animals were anesthetized by isoflurane inhalation, and a total volume of 0.5 mL of cell-loaded microcapsules (2×10^6 cells/mL) suspended in Hank's balanced salt solution (HBSS) was implanted subcutaneously using a 18-gauge catheter (Nipro Europe N.V., Belgium). Control animals received 1 mL of HBSS by the same route. Before implantation, microcapsules were washed several times in HBSS. Upon recovery, animals had access to food and water ad libitum. No immunosuppressant protocols were applied to the animals during this study.

Hematocrit Measurements. Blood was collected weekly by retro-orbital puncture using heparinized capillary tubes (Deltalab, Spain). Hematocrits were determined after centrifugation at 3000 rpm for 15 min of whole blood using a standard microhematocrit method. Results are expressed as mean ± standard deviation.

Histological Analyses. At day 130 after implantation, some animals were sacrificed, and microcapsules were retrieved and fixed in a 4% paraformaldehyde solution in 0.1 M sodium phosphate, pH 7.2. Serial horizontal cryostat sections (14 μm) were processed for hematoxylin−eosin staining.

Statistical Analyses. Data are presented as mean ± standard deviation. All statistical computations were performed using SPSS 11.0 (SPSS, Inc., Chicago, IL). Data between control and experimental groups were analyzed for statistical significance using Student's *t*-test according to the results of the Levene test of homogeneity of variances. A *P*-value of <0.05 was considered statistically significant.

III. Results and Discussion

Microcapsule Characterization. All cell-loaded microcapsules had a uniform and spherical morphology without irregularities on their surface as shown in Figure 1. Previous studies have reported the relevance of the materials employed in the elaboration of microcapsules to obtain biocompatible microcapsules.[25] However, not only the materials used but also the spherical and smooth shaped morphologies of the microcapsules have been observed to be of great importance to elude the host's immune response.[26] Furthermore, the fluorescence analysis of the microcapsules demonstrated the high viability

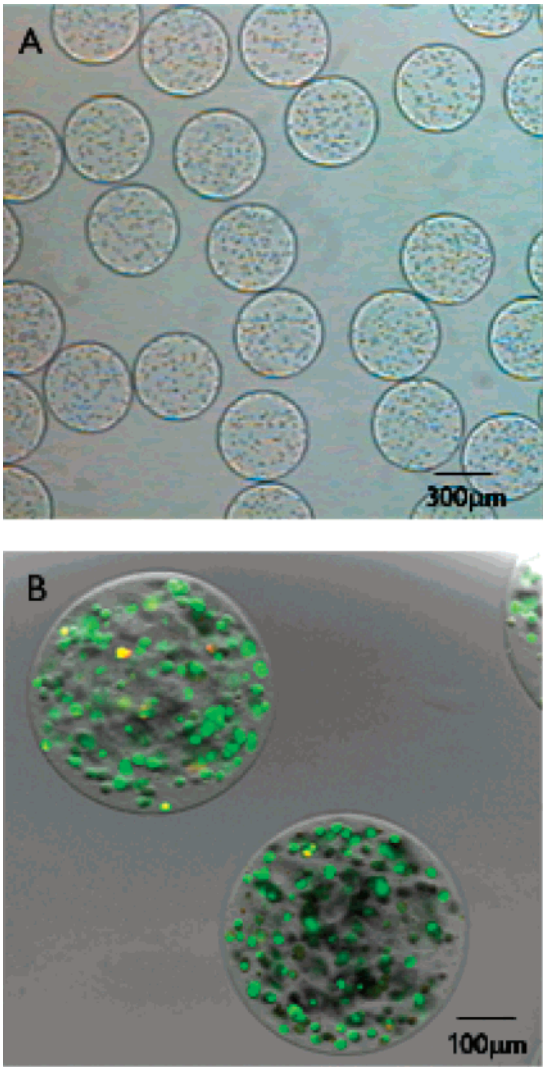

Figure 1. Morphologies of microencapsulated Epo-secreting myoblasts. (A) Optical microscopy. (B) Fluorescence image of cells stained with calcein-AM (green, live cells) and ethidium homodimer (red, dead cells).

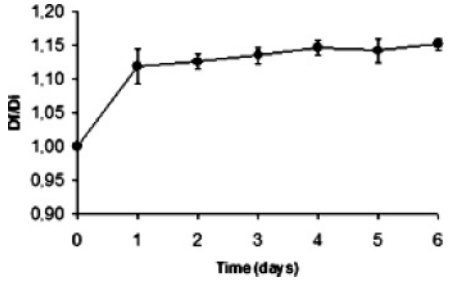

Figure 2. Osmotic pressure resistance of microcapsules after a 6 day treatment with citrate. The error bars on each point correspond to the standard deviation of the mean.

of the enclosed cells (Figure 1B), leading to the conclusion that enclosed cells were correctly adapted to the surrounding polymer scaffold.

Integrity and Stability of Microcapsules. Another important consideration is the study of the integrity and stability of the cell-loaded microcapsules. Alginates are nowadays the most frequently used biomaterials and generally present low immunogenicity, low toxicity, and thus good biocompatibility (which is one of the most important preconditions for biomaterials to be used clinically).[27] These positive features have made alginate

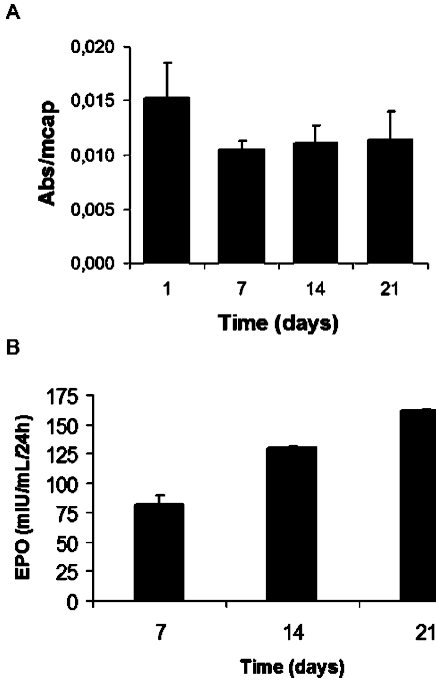

Figure 3. (A) In vitro viability of Epo-secreting C_2C_{12} myoblasts immobilized in APA microcapsules. (B) Epo secretion by entrapped cells (mIU/mL/24 h/100 microcapsules).

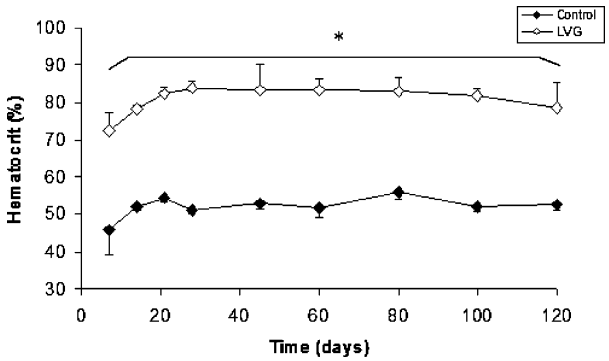

Figure 4. Hematocrit levels of Balb/c mice after subcutaneous implantation of Epo-secreting C_2C_{12} myoblasts immobilized in APA microcapsules. Values represent mean ± standard deviation. ∗ $P <$ 0.05 versus the control.

the polymer of choice in the field. Alginates create three-dimensional structures when they react with divalent cations such as calcium, barium, and strontium. Moreover, the election of adequate biomaterial compositions has been optimized by the combination of an alginate core surrounded by a polycation layer that at the same time is covered by an outer alginate membrane.[28] This microencapsulation design (alginate−polycation−alginate) is nowadays the most often described system in the scientific literature.[29] Alginate−PLL−alginate microcapsules have showed suitable mechanical strength and resistance to swelling in previous experiments carried out by our research group.[30]

In the present study, we performed a thorough in vitro characterization of the cell-loaded microcapsules to determine their suitability for the following in vivo assays. The mechanical resistance of the cell-loaded microcapsules against compression was 34.5 ± 7.5 g/microcapsule, which corroborates the membrane's resistance to bursting forces. On the other hand, the swelling assay showed that microcapsules swelled and increased their diameter by approximately 10% after the first citrate treatment, but afterward their size remained stable (Figure 2).

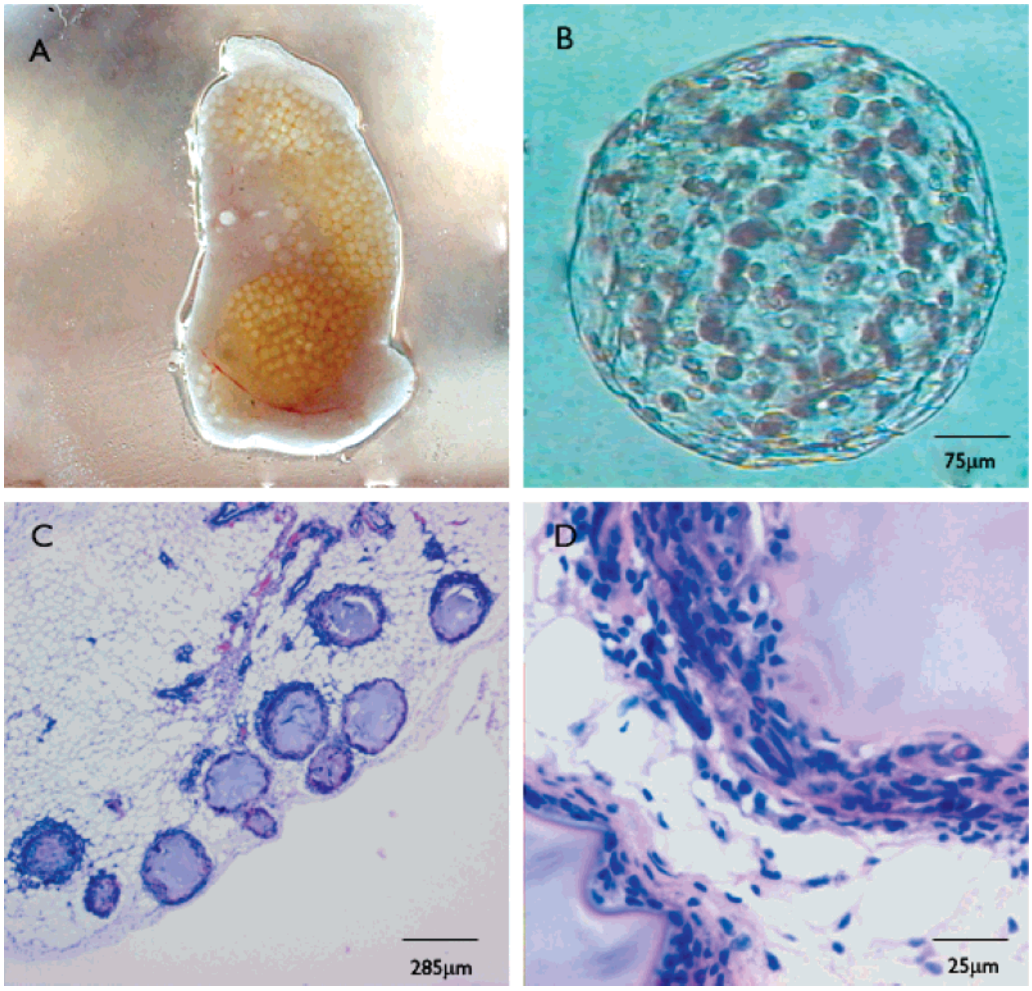

Figure 5. (A and B) Photographs of microcapsules explanted from the subcutaneous tissue 130 days post-implantation. (C and D) Histological analysis of explanted cell-containing microcapsules (hematoxylin−eosin).

These results confirm the high mechanical and chemical resistance of the microcapsules elaborated in this study.

Microencapsulation of C_2C_{12} Myoblasts: In Vitro Viability and Epo Production. The metabolic activity and Epo production of the encapsulated cells was analyzed over the course of 21 days. The level of viability detected is an indicator of the mitochondrial cell activity and therefore the physiological state of the enclosed cells. Thiazolyl Blue Tetrazolium Blue is a yellowish solution and is converted to water-soluble MTT−formazan of dark blue color by mitochondrial dehydrogenases of living cells. As seen in Figure 3A, C_2C_{12} myoblasts showed similar viabilities over the 21 days. A slight decrease was observed during the first week, but after this period entrapped cells maintained their viability, which supports the idea that the diffusion of nutrients and oxygen was not affected by an inappropriate membrane behavior.

C_2C_{12} myoblasts have been selected as a model cell line for immobilization by many research groups[31−33] in part due to the fact that they can be easily cultured and can afterward be terminally differentiated into myotubes[34] after immobilization both in vitro and in vivo.[35] Besides, myoblasts present a relative lack of major histocompatibility expression on the surface, which may lead to a decrease in the stimulation of humoral immune response.[36]

Regarding the cell line used in this study, a full characterization of the Epo production before and after encapsulation was carried out with the aim of adjusting the therapeutic dose of the microencapsulation product. The clone selected for immobilization released 46.3 ± 1.5 IU/mL Epo/10^6 cells/24 h. The reduction in the Epo release rate following encapsulation has been previously reported by our research group[23] observing a 66% reduction. Having a look at the Epo release by the immobilized cells (Figure 3B), the amount of Epo produced at day 7 by 100 cell-loaded microcapsules was 81.9 ± 8.2 mIU/mL/24 h. After this period, Epo release showed an important increase, reaching the highest production rate at day 21, 161.7 ± 0.9 mIU/mL/24 h. This could be explained by the fact that encapsulated myoblasts maintained in vitro were not induced to terminally differentiate into myotubes, leading to a slight increase in the overall cell density. In contrast, this induction into myotubes is promoted after the in vivo administration of the encapsulated cells, which enables a better control of the dosage.

Long-Term Hematocrit Levels of Balb/c Mice with Subcutaneously Implanted Epo-Secreting Microencapsulated Cells. On the basis of the in vitro Epo production, we estimated that 0.5 mL of cell-loaded microcapsules (2×10^6 cells/mL alginate) might result in a therapeutic dose to provide significant increase in mice hematocrit levels over time. To address this issue, adult female Balb/c mice were used as recipients, and cell-loaded microcapsules were implanted in the subcutaneous space. As it is observed in Figure 4, a significantly higher hematocrit level was observed in all the animals implanted with alginate microcapsules when compared with the HBSS (control) group ($P < 0.05$). Regarding the implanted group, the hematocrit levels of the animals increased to 84 ± 1.9% during the first 3

weeks of study, and afterward they remained asymptotic until day 120 post-implantation. Although the hematocrit level decreased slightly after this period, levels at day 120 post-implantation remained statistically high in comparison with the control group (78.7 ± 6.8% versus 52.5 ± 1.5%, respectively) ($P < 0.05$). However, a slight increase was also observed in the control group during the first 3 weeks followed by plateau until the end of the study.

Some interesting conclusions can be highlighted from these experiments. First, a 4 month release of Epo was observed after a single administration of cell-loaded microcapsules and following subcutaneous administration in allogeneic recipients. In previous studies, this route has been reported to result in poor implant viability and inconsistency in the hematocrit response to Epo secretion.[37] This long-term efficacy might be due to the optimized volume−surface relation of the microcapsules, which improves the cell product kinetics and oxygenation of the cells. Second, no remarkable side effects were observed during the treatment period although the high hematocrit levels obtained may be responsible for the appearance of polycythemia in the animals (expanded red cell mass).[38]

Microcapsule Retrieval and Histological Analysis. The implanted cell-loaded microcapsules from the treatment group were explanted at day 130 post-implantation. The macro- and microscopic appearances are shown in Figures 5A and 5B. Microcapsules retrieved from the subcutaneous tissue were mostly aggregated, forming an irregular structure in which immobilized cells remained viable. The microcapsule network was easily harvested as one piece after a small skin incision, as illustrated in Figure 5A. This could be an advantage, as one important challenge in the field of cell microencapsulation is the sometimes difficult removal of the implanted graft.

The histological analyses of the explanted microcapsules revealed the formation of some blood capillaries within the microcapsule aggregates. We hypothesized the latter could be due to the angiogenic effects reported for Epo. In fact, the Epo molecule has been reported to act as an angiogenic factor by different pathways.[39−41] This situation might be helpful as the access of oxygen and nutrients to the entrapped cells might be improved. Interestingly, although highly purified alginates were used for microcapsule elaboration and this process was done under aseptic conditions, a weak fibroblast overgrowth was detected surrounding the microcapsules (Figures 5C and 5D).

The data presented in this study demonstrate a proof-of-principle for cell encapsulation technology for the long-term delivery of Epo. The correct characterization of the immobilization systems and the genetically modified cell lines used are of paramount importance to optimize the final cell encapsulation product. Animals implanted with microencapsulated cells showed elevated hematocrit levels during 4 months of study, with no remarkable side effects. The presence at explantation of a cell-loaded microcapsule aggregate surrounded by several blood capillaries might be a consequence of the angiogenic effects of the Epo molecule. The latter may suggest the interesting role that this or any other type of angiogenic molecule could have in the long-term functionality of this type of cell-loaded microcapsules.

On the basis of the aforementioned advantages of this technology, this "living drug delivery system" can be considered as an alternative method for the systemic delivery of Epo from genetically engineered cells. Viable and functional cells will be able to release the desired products in a more physiological manner. Moreover, to overcome the current organ donor shortage, the immunoprotective properties of this device make this strategy suitable for allotransplantation therapy, turning this technology into an alternative therapy to whole organ transplantation.

Conclusion

In the present study, subcutaneous implantation of alginate−poly-L-lysine−alginate microcapsules containing Epo-secreting C_2C_{12} myoblasts in allogeneic mice recipients resulted in an important increase of hematocrit levels. High and constant levels were maintained over 120 days after a single administration of microcapsules and lacking immunosuppressive protocols. At explantation, a thin fibrotic layer was observed surrounding the microcapsules. The pharmacodynamic characteristics of Epo added to its poor pharmacokinetics make this molecule a candidate for its delivery using cell microencapsulation technology. Our results demonstrate that cell encapsulation technology might be a suitable therapeutic strategy for the long-term release of this therapeutic product.

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Supporting Information Available. Scheme of the cell encapsulation procedure using an electrostatic droplet generator. This material is available free of charge via the Internet at http://pubs.acs.org.

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