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Microcapsules prepared with different biomaterials to immobilize GDNF secreting 3T3 fibroblasts

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Abstract

Cell microencapsulation represents a promising tool for the treatment of many central nervous system (CNS) diseases such as Parkinson's disease. In this technology, cells are surrounded by a semipermeable membrane which protects them from mechanical stress and isolates them from host's immune response. However, if the future clinical application of this strategy is wanted, many challenges remain including the improvement of the mechanical resistance of the microcapsules and the optimization of the intracapsular microenvironment conditions. In this way, the selection of the matrix is essential because the morphological and the physiological behavior of the cells depend on the interactions between the matrix and the enclosed cells. Assuming these considerations, three types of microcapsules elaborated with four different polymers: alginate, cellulose sulfate, agarose and pectin have been fabricated and compared in order to evaluate some key properties such as morphology, size and mechanical stability. Furthermore, GDNF secreting Fischer rat 3T3 fibroblasts were immobilized in each type of capsule and the viability and neurotrophic factor release was determinated. Results showed that the alginate and pectin microcapsules were the most resistant devices, maintaining an adequate microenvironment for the enclosed cells. In contrast, cells entrapped in alginate–cellulose sulfate matrices presented the lowest mechanical resistance, cell viability and GDNF production.

Keywords: Microcapsules; Alginate; Pectin; Cellulose sulphate; Agarose; Glial cell line-derived neurotrophic factor (GDNF)

1. Introduction

Parkinson's disease is one of the major neurodegenerative disorders of middle and old age (Mayeux, 2003). It is characterized by a prominent loss of dopaminergic neurons in the substantia nigra pars compacta, resulting in a loss of dopaminergic innervation to the striatum (Agid, 1991). One approach for the treatment of this disease is based on the administration of the glial cell line-derived neurotrophic factor (GDNF) (Hefti, 1997). GDNF is a member of the transforming growth factor-β superfamily that has been shown to be

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neuroprotective and a survival factor for dopaminergic neurons of the substantia nigra (Yan et al., 1995). However, many problems have been found in the administration of this molecule because this factor does not cross the blood-brain barrier (BBB) so it has to be administered directly to the brain (Walton, 1999). This has stimulated the development of many approaches to deliver this molecule in an efficient way including brain infusion of GDNF by osmotic pumps (Lu and Hagg, 1997), encapsulation within PLGA microspheres (Jollivet et al., 2004), and in vivo and ex vivo gene therapy approaches (Choi-Lundberg et al., 1997; Shingo et al., 2002).

Cell microencapsulation is a promising tool for the treatment of central nervous system diseases such as Parkinson and it consists in surrounding cells with a semipermeable polymeric membrane. The latter permits the entry of nutrients and the exit of the therapeutic protein products, obtaining in this way a sustained delivery of the desirable molecule. The membrane isolates the enclosed cells from the host immune system, preventing the recognition of the immobilization cells as foreign. Therefore, a wide range of pharmacologically active cells can be encapsulated, leading to sustained and localized delivery of small doses of the therapeutic molecule directly into the brain (Lysaght and Aebischer, 1999).

However, if the long-term viability of the enclosed cells is achieved, some technological challenges must be addressed. In particular it is necessary to develop optimized microcapsules with an optimal membrane geometry and morphology which allow the correct transport of molecules into and out of the device. In addition, the matrix structure must be optimized since the interactions between the matrix and the embedded cells might play an important role in their morphological and physiological behavior (Bienaimé et al., 2002). For example, adrenal chromaffin cells have been immobilized in alginate to prevent aggregation, which reduces the formation of central necrotic cores. In contrast PC12 cells exhibit a preference for distribution within precipitated chitosan, which provides a better scaffolding structure (Emerich et al., 1993). Assuming this, many polymers including alginate, rat tail collagen extracts, gelatin shards, chondroitin sulfate, and chitosan (Emerich and Winn, 1999) have been employed in cell microencapsulation technology.

In this paper, we have prepared different beads with varied proportions of polymers such as alginate, cellulose sulfate, agarose and pectin and we have evaluated their physical, morphological and mechanical properties. Homogeneous and non-homogeneous polymeric matrices were produced by controlling the kinetics of the gel formation, leading to gels with varying degrees of anisotropy with respect to polymer concentration. The compression resistance, swelling behavior and geometry of each matrix as well as their effect over the viability and GDNF secretion of encapsulated Fischer rat 3T3 fibroblasts have been studied. The final aim is to fabricate an optimized polymeric system for the long-term secretion of GDNF to be employed in the treatment of Parkinson's disease.

2. Materials and methods

2.1. Materials and cells

Alginate LVG, low viscosity alginate with a high content in guluronic acid, was obtained from FMC Biopolymers (Norway). Agarose type IX, gelling at 8–17 °C and remelt at <50 °C, poly-L-lysine hydrobromide (PLL, MW: 29,300) and sodium pectin, were obtained from Sigma (St. Louis, MO, USA). Cellulose sulfate was purchased from Acros Organics (Geel, Belgium) and the remaining chemicals were obtained from Sigma (St. Louis, MO, USA).

Fischer rat 3T3 fibroblasts transfected to produce GDNF, were kindly donated by the Laboratory of molecular neurobiology, Karolinske Institute, Stockholm. Cells were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum, 1 mg/ml L-glutamine, 1 mg/mL penicillin–streptomycin, and 200 μg/ml geneticine (Arenas et al., 1995). The cells were maintained in culture at 37 °C in a humidified 5% CO_2/95% air atmosphere and passaged every 2–3 days. All reagents were purchased from Gibco BRL (Invitrogen, Spain).

2.2. Microcapsule elaboration

Different types of matrices, homogeneous and non-homogeneous, were elaborated using an electrostatic droplet generator. The non-homogeneous matrices are

characterized by a higher concentration of alginate in the surface than the homogeneous matrices (Orive et al., 2003a). In addition, two different potentials, 400 and 800 kV, were used to obtain microbeads of different diameters, around 1.7 mm for the compression resistance test and around 500 μm for the rest of the studies.

In the elaboration of the different types of microcapsules, a 2% (w/v) alginate solution was mixed with the other polymer solutions: agarose type IX 2% (w/v), cellulose sulfate 2% (w/v) and sodium pectin 2% (w/v) in different proportions (1:1, 1.25:0.75, 1.5:0.5, 1.75:0.25). Polymer mixtures were extruded into 55 mM anhydrous calcium chloride solution (for non-homogeneous microcapsules) or 55 mM anhydrous calcium chloride solution with 0.9% sodium chloride (for homogeneous microcapsules). The microbeads obtained were maintained in agitation for 5 min in the solution of calcium chloride in order to complete the ionic gelation. The polymerization process of microbeads elaborated with alginate and agarose include an ionic and thermal gelation due to the presence of agarose, which polymerizes at 8–17 °C, while the other two mixtures required only an ionic gelification process. Finally, the microbeads were chemically cross-linked with 0.05% (w/v) PLL for 5 min and then with another layer of 0.1% (w/v) alginate for 5 min.

Fischer rat 3T3 fibroblasts were immobilized within non-homogeneous microcapsules. Briefly, Fischer rat 3T3 fibroblasts (5×10^6 cells per ml) were suspended in the polymer mixture composed of a combination of 1.5:0.5 (alginate–other polymer). The cell-gel suspension was extruded into 55 mM anhydrous calcium chloride solution and the beads were coated as explained above.

2.3. Effect of the concentration of calcium chloride on the formation of the beads

Since alginates polymerize in the presence of calcium ions, the effect of the concentration of such ions play a key role in the formation and in the size of the non-homogeneous and homogeneous microbeads. In this assay, we evaluated the influence of calcium chloride concentration in the fabrication of microbeads with a polymer proportion of 1.5:0.5 (alginate–other polymer). A range of calcium chloride concentrations were studied: 25, 50, 75, 100, 200 mM (with 0.9% of sodium chloride for homogeneous microbeads).

2.4. Microbead and microcapsule characterization

A uniform shape and a smooth and regular microcapsule membrane is essential to ensure the viability of the microencapsulated cells and the biocompatibility of the immobilization devices (De Vos et al., 1994). Therefore, a complete morphological characterization was carried out using an inverted optical microscope (Nikon TMS) equipped with a camera (Sony CCD-Iris).

2.5. Mechanical stability studies

2.5.1. Compression resistance study

The effect of plasma on microcapsule compression resistance was evaluated by incubating approximately 30 capsules in a 10 mL plasma solution medium (40% (v/v) in PBS) and shaking the capsule suspension at 800 rpm for 30 min. Afterwards, the compression resistance of the microbeads and microcapsules was determined as the main force (g) required to generate a 75% compression of the sample using a Texture Analyzer (Model TA-T2i, Stable Microsystems, Surrey, UK). Results are expressed as mean ± S.D. for 10 replicates.

2.5.2. Swelling study of the microcapsules

This assay is a modification of one elaborated by our group for testing alginate–PMCG–alginate microcapsules (Orive et al., 2003b). Briefly, 100 μl of microcapsules (around 50 microcapsules) of a known diameter were mixed with 900 μl of saline solution (PBS) and placed in a 24-well cell culture cluster which was put in a shaker at 800 rpm and 37 °C for 1 h. Afterwards, 800 μl PBS was eliminated and replaced by 800 μl of 1% sodium citrate or purified water and the cluster was maintained at 37 °C for 24 h. The following day the diameter of 20 microcapsules of each type was measured and the number of the broken microcapsules was counted. The washing and shaking step with PBS and the static conditions with each treatment were repeated during the following 9 days. Results are expressed as (D_f/D_i), where D_i and D_f are the diameter of the capsule before and after the treatment, respectively.

2.6. Determination of the viability of the enclosed cells

The viability of the microencapsulated cells was determined by the tetrazolium assay (Uludag and Sefton, 1990). In summary, 25 μl of 5 mg/ml solution of MTT in PBS was added into around 40 microcapsules placed in a 96-well cell culture cluster and incubated at 37 °C for 4 h. Afterwards, the MTT solution was removed by vacuum aspiration and 100 μl of dimethylsulphoxide was added. The purple solution was read 5 min later on a microplate reader (Titerteck Multiscan Lab Systems) at 560 nm test wavelength with reference at 690 nm. Results are expressed as mean ± S.D. for three replicates.

2.7. GDNF production

The production of GDNF by the microencapsulated cells was measured using the GDNF E_{max}® Immunoassay system (Promega, Madison, USA) which detects GDNF in the range of 16–1000 pg/ml. Around 100 microcapsules of each type were put in a 24-well cell culture cluster with 500 μl of the culture medium and maintained them in the normal culture conditions for 24 h. Afterwards, the media was collected and undiluted samples were assayed for GDNF content. Results are expressed as mean ± S.D. for three replicates.

2.8. Statistical study

The results of this study are expressed as mean ± S.D. The Student's *t*-test was used to detect significant differences when two groups were compared. One-way ANOVA and post-hoc test were used in multiple comparisons. The Scheffé or Tamhane post-hoc test was applied according to the result of the Levene test of homogeneity of variances. All statistic computations were performed using SPSS 11.0 (SPSS, Inc., Chicago, IL).

3. Results and discussion

Microcapsules with an internal homogeneous and non-homogeneous matrix produced combining alginate and another polymer have been elaborated. Materials widely used in microencapsulation have been selected including pectin (Navrátil et al., 2000), cellulose sulfate (Ceausoglu and Hunkeler, 2002) and agarose (Iwata et al., 1988) Therefore, alginate–pectin (AP), alginate–cellulose sulfate (AC) and alginate–agarose (AA) microcapsules have been fabricated in order to compare the stability and functionality of the matrices.

3.1. Characterization of microbeads prepared with different polymer proportions

Homogeneous and non-homogeneous AA, AP and AC microbeads elaborated with different proportions of each material (1:1, 1.25:0.75, 1.5:0.5, 1.75:0.25; alginate:other material) were prepared and characterized before microcapsule fabrication.

3.1.1. Microbeads size

As is shown in Fig. 1A and B all microbeads have been characterized with the exception of alginate and pectin (1:1) beads, because it was impossible to obtain microbeads with spherical and good morphology. In general, the non-homogeneous beads showed a smaller diameter than the homogeneous ones ($P < 0.05$), with the exception of AC in the proportion 1:1 and AA in the proportion 1.5:0.5. Interestingly, the diameter of AA microbeads decreased when the proportion of alginate was higher while in the case of AP and AC beads the size of the matrices increased with the alginate proportion. This could be a consequence of the different polymerization processes of the polymers, in which agarose is the only one that does not shrink with calcium ions.

3.1.2. Compression resistance study of the microbeads

To address the problem of matrix type selection, a compression resistance study was carried out assuming the importance of microcapsule mechanical stability in the design of cell immobilization devices for therapeutic purposes. Fig. 2A and B shows the main force (g) required for 75% compression of one microbead. Resistance against compression was slightly higher in the case of non-homogeneous matrices than homogeneous ones with ($P < 0.05$) when the proportion of the secondary material was high (1:1). This behavior has been reported by our group for other types of microcapsules (Orive et al., 2003a).

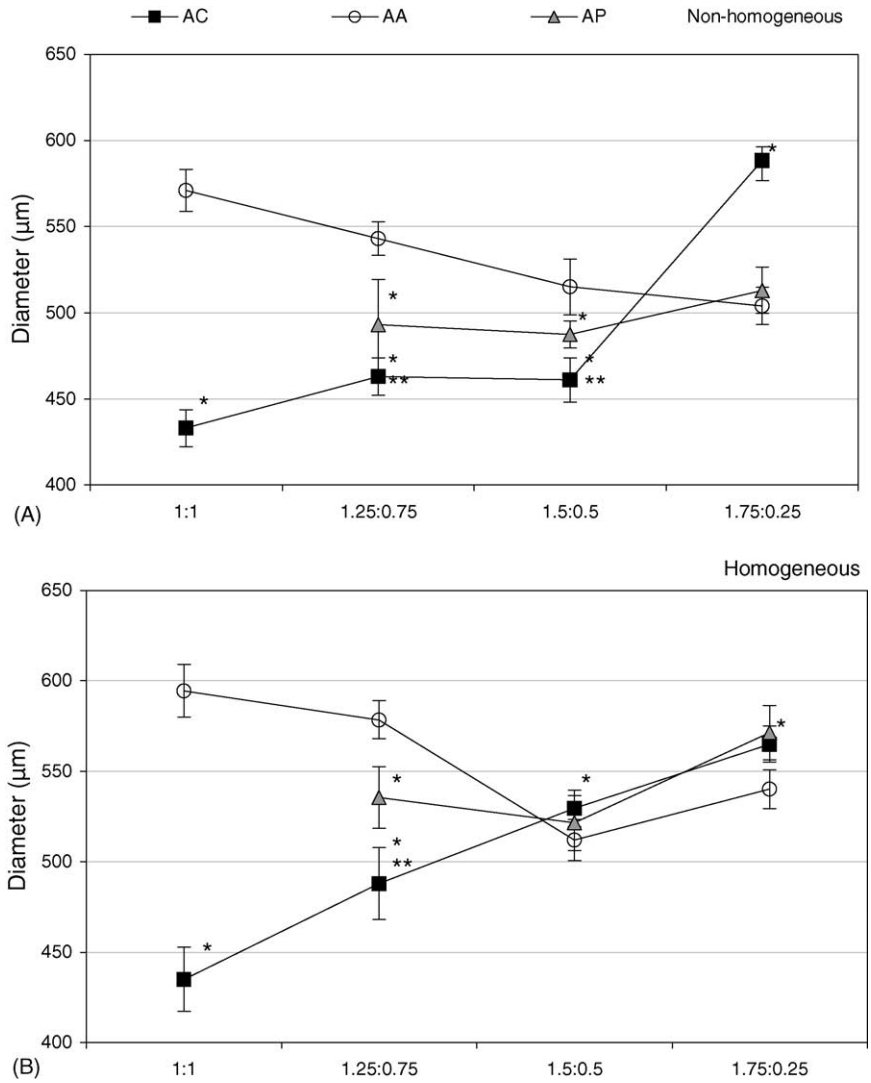

Fig. 1. Diameter of the non-homogeneous microbeads (A) and homogeneous microbeads (B) elaborated with different proportions of alginate–cellulose sulfate (AC), alginate–agarose (AA), and alginate–pectin (AP). $^{*}P < 0.05$ vs. AA, $^{**}P < 0.05$ vs. AP in each proportion and in each different configurations. Statistically significant differences between non-homogeneous and homogeneous with the exception of AC (1:1) and AA (1.5:0.5).

AA microbeads showed the smallest resistance ($P < 0.05$), at all the proportions and configurations studied with the exception of 1.5:0.5 proportion. In addition, a higher proportion of alginate in the matrix increased the compression resistance of the system due to the increased interaction with the gelifying calcium ions which leads to the generation of gels with stronger mechanical properties.

3.2. Effect of calcium chloride concentration

The concentration of calcium chloride used is an important parameter in the elaboration of the microcapsules. Although the most frequent employed calcium chloride concentrations are 100 mM $CaCl_2$ (De Haan et al., 2003) and 50 mM $CaCl_2$ (Klokk and Melvik, 2002), it is necessary to determine which concentra-

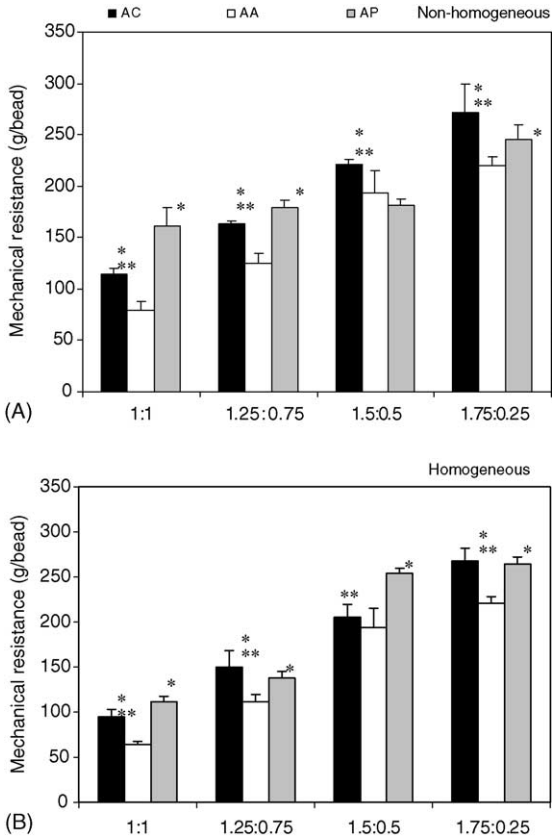

Fig. 2. Mechanical resistance of non-homogeneous (A) and homogeneous (B) microbeads prepared with different polymer proportions (1:1, 1.25:0.75, 1.5:0.5, 1.75:0.25). $^{*}P < 0.05$ vs. AA, $^{**}P < 0.05$ vs. AP in each proportion and in the different configurations. Statistically significant differences between non-homogeneous and homogeneous with the exception of AA (1.25:0.75, 1.5:0.5, 1.75:0.25) and AC (1.25:0.75, 1.5:0.5, 1.75:0.25).

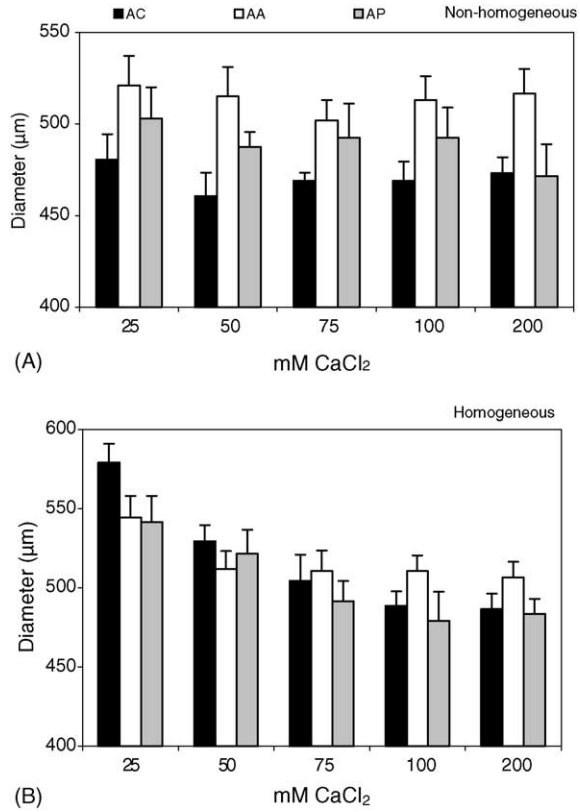

Fig. 3. Effect of the concentration of calcium chloride in the diameter of non-homogeneous (A) and homogeneous (B) microbeads.

tion of calcium chloride provides a better formation of the microbeads. To address this issue, we fabricated AA, AP and AC microbeads in a proportion 1.5:0.5 with different concentrations of calcium chloride. Results showed that diameters of all the non-homogeneous beads remained constant at the different calcium chloride concentrations while in the case of homogeneous beads the size decreased as the concentration of the gelifying agent increased (Fig. 3A and B). This could be a consequence of the tightening of the gel network with the increased calcium concentrations resulting in smaller microbeads (Klokk and Melvik, 2002). However, in the case of non-homogeneous microcapsules the penetration of calcium ions is limited by the dense "membrane" of very high alginate concentration at the calcium chloride/alginate interface (Skjåk-Braek et al., 1989). Interestingly, it was observed that non-homogeneous AA microbeads presented the largest size ($P < 0.05$), in all the calcium chloride concentrations with the exception of 75 mM concentration in which there was not statistically significant differences between the AA microbeads and AP microbeads.

A 50 mM calcium chloride concentration was selected in part because this concentration enables the elaboration of microbeads with a good morphology and avoids the use of higher calcium chloride concentration. The latter could cause some problems in terms of viability and functionality of the enclosed cells and would increase the permeability of the gel. In fact, at high concentrations of calcium chloride, more than

two G-block sequences take part in the junction zones which leads to more space between the junction zones making a more porous gel (Gåserød et al., 1998).

3.3. Compression resistance study of the microcapsules

According to the results obtained, the polymer combination (1.5:0.5; alginate:other material) was selected for preparing microcapsules using 50 mM calcium chloride concentration as polymerizing agent and PLL and alginate as coating membranes. Fig. 4A shows the main force (g) required for 75% compression of non-homogeneous and homogeneous microcapsules.

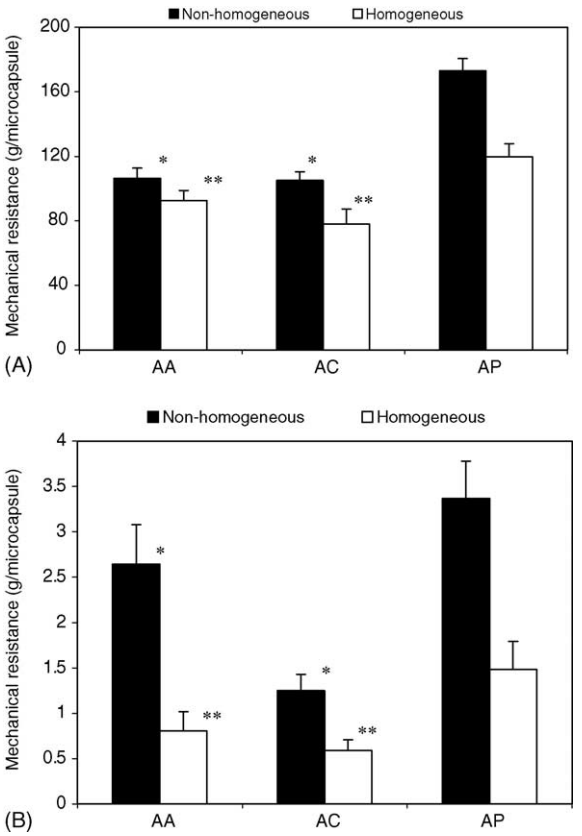

Fig. 4. Mechanical resistance of non-homogeneous and homogeneous microcapsules (1.5:0.5) before (A) and after (B) exposure to a solution of plasma for 30 min. Differences statistically significant between non-homogeneous and homogeneous for each matrix. $^{*}P < 0.05$ vs. AP non-homogeneous. $^{**}P < 0.05$ vs. AP homogeneous.

As it is observed, non-homogeneous microcapsules resulted in more resistance than homogeneous ones while AP matrices, homogeneous and non-homogeneous, showed the highest compression resistance ($P < 0.05$). This could be caused by the gelation process of the pectin which is similar to the alginate (Lootens et al., 2003) leading to a higher accumulation of Ca^{2+} within the gel, a fact that is correlated with the gel strength. The latter is particularly important because a good mechanical stability might improve and prolong the in vivo performance of the encapsulated cells (Van Raamsdonk and Chang, 2000). When the different types of matrices were incubated in destabilizing plasma solutions, a potent reduction in the stability properties of the devices was detected (Fig. 4B). These results agree with those obtained by Van Raamsdonk et al. (2002). AP microcapsules resulted again in the most resistant matrices while AC capsules were the weakest ($P < 0.05$). Furthermore, the higher concentration of alginate in the periphery of the microcapsules result in higher resistance values for non-homogeneous microcapsules ($P < 0.05$).

3.3.1. Swelling study of the microcapsules

The osmotic resistance of the stronger non-homogeneous microcapsules was evaluated using a swelling test both with purified water and 1% (w/v) sodium citrate solution. The former induces the entrance of water within the capsule by an osmotic phenomenon (Fig. 5A and C) while the latter provokes exchange of the strongly bound cross-linking calcium ions with non-gelling sodium ions, giving as a result an increase in the capsule diameter. Results showed that AC microcapsules suffered a slightly higher increase ($P < 0.05$) in their diameter (Fig. 5B and D) when sodium citrate was used, a feature that might be explained by the instability of the calcium–cellulose sulfate interaction in the presence of sodium ions (Lacík et al., 1998). Furthermore, when the number of broken microcapsules after incubation with both mediums was measured, it was observed that although the percentage of broken AA and AP microcapsules remained small, an important 70% and 50% of broken AC microcapsules were detected for citrate solution and water, respectively (Fig. 5E). This gave an idea of the lowest tensile resistance offered by the capsules elaborated with an alginate–cellulose sulfate matrix ($P < 0.05$).

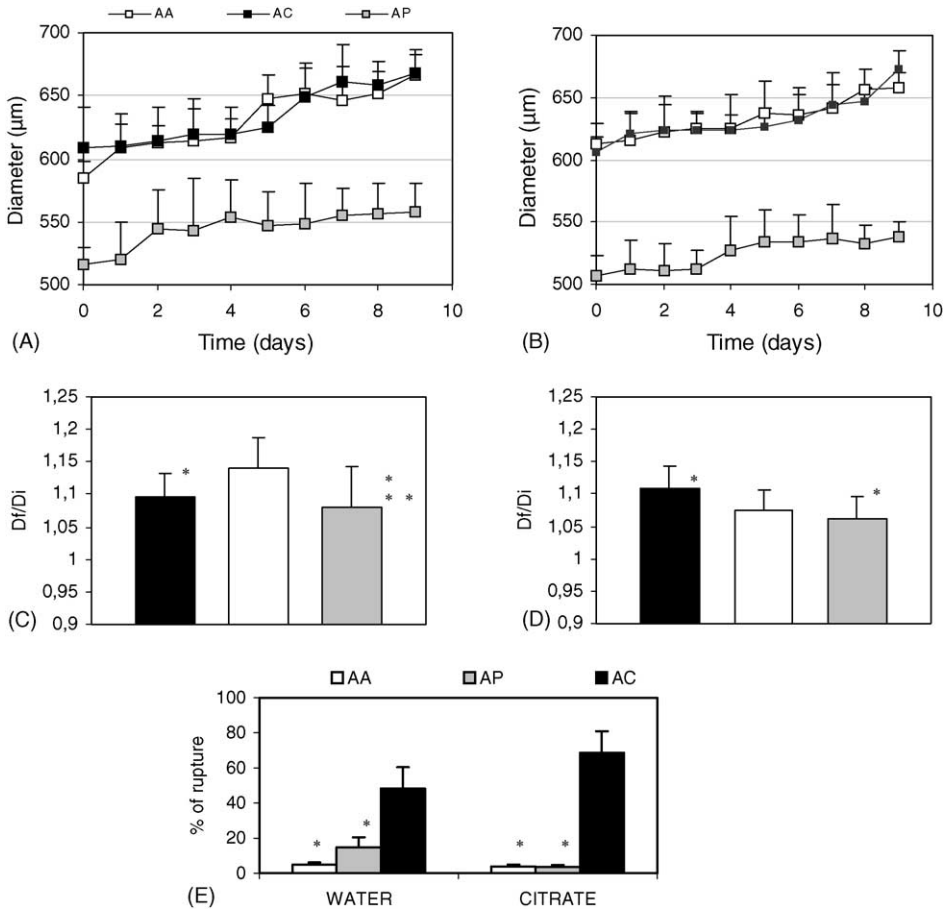

Fig. 5. Effect of the washing with water (A) or 1% citrate (B) in the diameter of the non-homogeneous microcapsules. Relations between the initial diameter (D_i) and the final diameter(D_f) after nine days of washing with water (C), $^{*}P < 0.05$ vs. AA, $^{**}P < 0.05$ vs. AC, and 1% citrate (D), $^{*}P < 0.05$ vs. AA. Percent of rupture of the different microcapsules in different mediums (E). $^{*}P < 0.05$ vs. AC.

3.4. Cell microencapsulation: viability and GDNF production study

Non-homogeneous microcapsules elaborated with a matrix composed by alginate–pectin showed the best mechanical properties. Nevertheless, we still needed to demonstrate what type of matrix allowed the better viability and functionality of the immobilized cells. To address this issue, GDNF secreting Fischer rat 3T3 fibroblasts were encapsulated within the different types of polymer matrices and maintained in culture for 20 days. Microscopically Fischer rat 3T3 fibroblast cells seemed to grow better in AA and AP matrices while in the case of AC microcapsules, cells were created with some small and dark aggregates, probably as a consequence of the poor mass transfer of oxygen to the interior of the aggregates (Fig. 6).

The metabolic activity of the immobilized cells showed a distinctive behavior in each type of matrix (Fig. 7A). Cells entrapped in AP and AA microcapsules showed a higher metabolic activity that cells immobilized in AC capsules, a difference that was statistically significant ($P < 0.05$) for 6-, 9- and 20-day time-period. These results are in accordance with the previous microscopic evaluation of the encapsulated cells. Furthermore, some AC microcapsules broke during the study, releasing part of their immobilized cell-

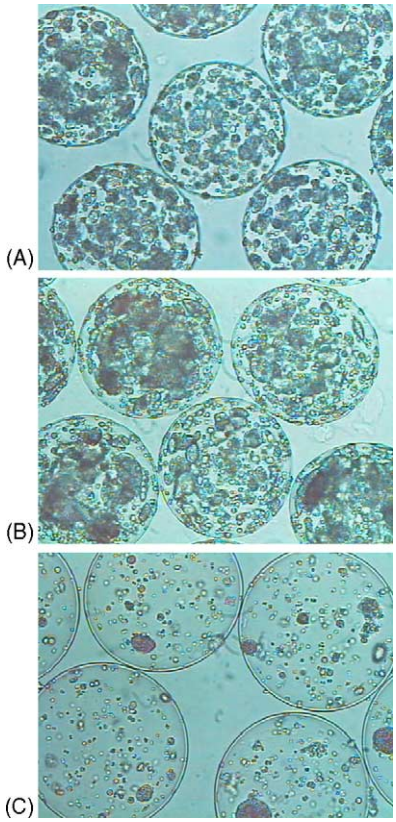

Fig. 6. Morphology of microencapsulated GDNF secreting Fischer rat 3T3 fibroblasts in different microcapsules: alginate–agarose (A), alginate–pectin (B) and alginate–cellulose sulfate (C), in the days 1, 8, 14 and 20 of culture.

dose. We then investigated if GDNF production would correlate with the viability results of the encapsulated cells. Therefore, the GDNF secretion from the immobilized cells was measured using an immunoassay. In the case of immobilized cells, results indicated that cells encapsulated in AP and AA microcapsules secreted higher levels of GDNF ($P < 0.05$) than cells entrapped in AC matrices both at 15 and 22 days post-encapsulation (Fig. 7B). GDNF production by the cells enclosed in AC microcapsules at 15 day was lower than detection limit. These observations support the idea that polymer type selection may play a major role in the activity of the enclosed cells and consequently in the long-term functionality of this therapeutic strategy.

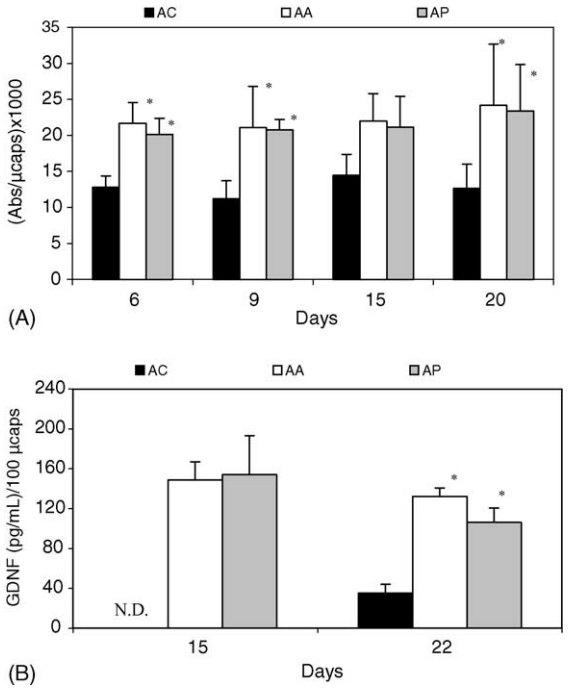

Fig. 7. (A) Viability of GDNF secreting Fischer 3T3 rats fibroblasts in the different matrices. $^{*}P < 0.05$ vs. AC. (B) GDNF secretion by cells entrapped in 100 microcapsules. N.D.: not detectable. $^{*}P < 0.05$ vs. AC.

4. Conclusion

Careful selection of microcapsule materials is necessary if mechanically resistant microcapsules and long-term cell viability and GDNF secretion are required. In this work, we show how the type of polymer mixture exerts a direct effect on the compression resistance and osmotic swelling behavior of the capsules as well as on the functionality of the enclosed cells. Results indicated that AP matrices constitute the most resistant microcapsules and that this stronger gel network does not impede the entrance of oxygen and nutrients as well as the exit of GDNF. In contract, AC microcapsules were observed to be the most fragile ones according to the compression resistance study and the percentage of broken capsules after exposure to plasma. Furthermore, cells immobilized in AC capsules showed a very reduced GDNF production and metabolic activity. In summary, selection and elaboration of suitable polymer-matrices is essential if long-term treatments with encapsulated products are achievable.

Acknowledgements

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