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Graphene oxide increases the viability of C_2C_{12} myoblasts microencapsulated in alginate

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ABSTRACT

Cell microencapsulation represents a great promise for long-term drug delivery, but still several challenges need to be overcome before its translation into the clinic, such as the long term cell survival inside the capsules. On this regard, graphene oxide has shown to promote proliferation of different cell types either in two or three dimensions. Therefore, we planned to combine graphene oxide with the cell microencapsulation technology. We first studied the effect of this material on the stability of the capsules and next we analyzed the biocompatibility of this chemical compound with erythropoietin secreting C_2C_{12} myoblasts within the microcapsule matrix. We produced 160 μm-diameter alginate microcapsules with increasing concentrations of graphene oxide and did not find modifications on the physicochemical parameters of traditional alginate microcapsules. Moreover, we observed that the viability of encapsulated cells within alginate microcapsules containing specific graphene oxide concentrations was enhanced. These results provide a relevant step for the future clinical application of graphene oxide on cell microencapsulation.

1. Introduction

Cell microencapsulation is widely used in research as a delivery system for therapeutic drugs. The structure of the capsule enables the flow of nutrients and gases (O_2 and CO_2) between its outer and inner side, as well as the waste outlet and release of therapeutic molecules produced *de novo* by encapsulated cells. The aim is to create a suitable microenvironment that promotes and controls cell viability and proliferation. Furthermore, microcapsules protect the cells from the immunological rejection they would suffer from if cells were directly implanted in an immunocompetent host. In fact, microcapsules do not allow the entrance of immunoglobulins or the recognition of the cells by the immune system. (Orive et al., 2013)

Different biomaterials such as agarose (Dang et al., 2004), chitosan (Li et al., 2010) or hyaluronic acid (Gerecht et al., 2007) have been used for cell microencapsulation. However, alginate (Siti-Ismail et al., 2008) remains the most common choice for the development of such systems due to its excellent biocompatibility and suitable mechanical properties. Pancreatic islets encapsulation in alginate matrices is the field in which cell microencapsulation more progress has made, with promising results starting in the early 90s. Encouragingly, one patient got to be independent of insulin injections for 9 months thanks to the intraperitoneal administration of pancreatic islets encapsulated in alginate (de Vos et al., 2006; Lim and Sun, 1980; Soon-Shiong et al., 1994). Currently, clinical trials are assessing the use of encapsulated xenogeneic islet transplants and they are showing an adequate biosafety profile

Abbreviations: A549, human lung carcinoma epithelial cell line; AFM, atomic force microscopy; BSA, bovine serum albumin; CCK-8, cell counting kit-8; C2C12, mouse myoblast cell line; EPO, erythropoietin; FT-IR, Fourier transform infrared; GO, graphene oxide; LDH, lactato deshidrogenase; PLL, poly-L-lysine; QI, quantitative imaging; SEM, scanning electron microscope.

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together with high effectiveness in patients with type I diabetes mellitus (Basta et al., 2011; Calafiore et al., 2006). Our research group has previously studied the microencapsulation of genetically modified C_2C_{12} myoblasts to secrete erythropoietin (EPO), and showed that the encapsulation of these cells allows the release of EPO for at least 210 days in an allogeneic transplant and for 98 days in a xenogeneic transplant (Murua et al., 2009; Orive et al., 2005).

In spite of the great promise of cell encapsulation technology, there have been continuous challenges in cell therapy using microencapsulated cells. The major one has been to decrease the number of dying cells inside the microcapsules. In fact, these cells release factors that are detrimental to the health of neighboring cells. Moreover, they can be released to the host environment eliciting an antigenic response (Lim et al., 2010). Thus, the search of new materials that can enhance microencapsulated cells viability is one the most important current trends on cell microencapsulation field.

Research on graphene, a two dimensional sheet with exceptional electrical, mechanical and thermal properties, has grown exponentially since its discovery. Its application in electronic and optoelectronic systems, in medicine, as photoconductive materials and delivery systems of therapeutic drugs, or even in tissue engineering has been explored (Goenka et al., 2013). Similarly, the application of nanomaterials from graphene derivatives, such as graphene oxide, has been studied. Graphene oxide (GO) is a chemically modified highly oxidized form of graphene, consisting of single atom thick layer of graphene sheets with carboxylic acid, epoxide and hydroxyl groups (Goenka et al., 2013). Thus, for example, surfaces of graphene and GO have shown to support proliferation and differentiation of induced pluripotent stem cells, promoting differentiation toward endoderm lines such as the insulin producing cells (Chen et al., 2012). Moreover, graphene and GO increase the adhesion, proliferation and differentiation of mesenchymal stem cells toward osteogenic and adipogenic lines respectively (Lee et al., 2011; Nayak et al., 2011). GO has also shown a higher rate of maturation from murine myoblasts into myotubes compared with other materials (Ku and Park, 2013). Although most of the studies reported are performed on two dimensional (2D) graphene materials, three-dimensional (3D) scaffolds have also been studied. For instance, graphene foams support neural stem cell (NSC) proliferation and differentiation toward neuronal lineages (Li et al., 2013a). 3D composite scaffolds have also been fabricated with gelatin methacrylate and GO, enhancing gelatin methacrylate mechanical and electrical properties, while not affecting the viability of encapsulated fibroblast cells (Shin et al., 2013). Overall, graphene materials, either on 2D or 3D, seem to promote the proliferation of different cell types although more detailed *in vitro* characterization of scaffolds needs to be done.

Because of the unique properties of GO, we propounded to combine cell microencapsulation technology with this material. In fact, the selection of suitable polymer-matrices is essential to achieve long-term treatments with encapsulated cells. For example, alginate and pectin microcapsules have shown more resistance and maintenance of an adequate microenvironment for enclosed GDNF secreting Fischer rat 3T3 fibroblasts than the combination of alginate with other materials, such as cellulose sulfate or agarose (Ponce et al., 2005). Currently, there is only one study that has combined alginate capsules with GO. In this study, the size of alginate beads containing GO was 50 millimeters of diameter, suggesting the application of these spheres as efficient copper adsorption material (Algothmi et al., 2013). Our aim was to incorporate different concentrations of GO into 160 μm-diameter alginate microcapsules, characterize their physical properties and test its biocompatibility with EPO producing C_2C_{12} myoblasts within the microcapsules for future clinical applications.

2. Material and methods

2.1. Biomaterials

Graphene oxide (GO) 5 wt% was obtained from Graphenea Company (Spain). The product was suspended in water and sonicated for 1 h in order to obtain a higher percentage of monolayer flakes. Ultra pure low-viscosity and high glucuronic acid alginate (LVG) was purchased from FMC Biopolymer (Norway). Poly-L-lysine hydrobromide (PLL, 15–30 kDa) was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Size, PDI and zeta potential measurements

The size and the poly-dispersity index (PDI) were determined by dynamic light scattering (DLS), and the superficial charge by laser doppler velocimetry (LDV) on a Zetasizer Nano ZS (Malvern Instruments, UK). Samples were diluted in Mili-Q water and the particle size, reported, was obtained by cumulative analysis. All measurements were carried out in triplicate.

2.3. Cell culture

Murine C_2C_{12} genetically engineered to secrete murine erythropoietin (C_2C_{12}–EPO) (Murua et al., 2009; Orive et al., 2005) were grown in T-flasks with Dulbecco's modified Eagles's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco) and 1% antibiotic/antimycotic solution (Gibco) at 37 °C in humidified 5% CO_2/95% air atmosphere. Cells were passaged every 2–3 days.

2.4. Cell microencapsulation

Sterile 1.87% alginate solutions were prepared by dissolving LVG alginate in a 1% manitol solution and filtering through a 0.20 μM syringe filter (Millipore, MA, USA). GO was suspended in sterile water at a concentration of 5 mg/ml and sonicated for 1 hour. Next, the following concentrations were prepared by dilution with sterile water:, 2,5 mg/ml, 1,25 mg/ml, 1 mg/ml, 625 μg/ml, 500 μg/ml, 250 μg/ml, 125 μg/ml and 50 μg/ml. GO suspensions were mixed with 1.87% alginate solutions to a final concentration of 1.5% alginate and the following GO final concentrations: 1 mg/ml, 500 μg/ml, 250 μg/ml, 200 μg/ ml, 125 μg/ml, 100 μg/ ml, 50 μg/ml, 25 μg/ml and 10 μg/ml. These suspensions were extruded through a disposable nebulizer using a 5 ml sterile syringe in a pneumatic atomization generator (Bioencapsulation portable platform Cellena®). The resulting alginate beads were maintained in agitation for 10 min in a $CaCl_2$ solution (55 mM) for complete ionic gelation. Afterwards, they were ionically linked with 0.05% (w/v) PLL for 5 min and coated with 0.1% LVG alginate for another 5 min. Microcapsules were prepared at room temperature, under aseptic conditions and were cultured in C_2C_{12} medium.

For cell microencapsulation with GO, murine C_2C_{12}–EPO cells were harvested using 0.25% trypsin–EDTA (Life Technologies, Carslbad, CA) and resuspended into sterile 1.5% alginate solutions containing the following final GO concentrations: 200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml and 10 μg/ml. As controls, C_2C_{12}–EPO cells were resuspended in a 1.5% alginate solution without GO. Cells were encapsulated at a density of 4×10^6 cells/ml of alginate. The diameters (160 μm) and overall morphology of microcapsules were characterized using an inverted optical microscopy (Nikon TSM).