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Brief communication

Thermosensitive polymeric matrices for three-dimensional cell culture strategies

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ABSTRACT

A completely new strategy for cell culture focusing on the design of three-dimensional (3D) smart surfaces by supercritical fluid technology has been developed. This approach might overcome the limitations on cell expansion and proliferation of currently existing techniques. An alternative technology, based on supercritical carbon dioxide, was used to polymerize poly(N-isopropylacrylamide) (PNIPAAm) and to foam poly(D,L-lactic acid) (P_{D,L}LA), creating a thermosensitive 3D structure which has proven to have potential as a substrate for cell growth and expansion. We demonstrated that the thermosensitive matrices promoted cell detachment, thus P_{D,L}LA scaffolds have the potential to be used as substrates for cell growth and expansion avoiding enzymatic and mechanical methods of cell harvesting. The harvested cells were replated to evaluate their viability, which was not compromised. A major advantage of this technology is the fact that the prepared materials can be recovered and reused. Therefore, the same substrate can be recycled and reused for different batches. An indirect impact of the technology developed is related to the field of biotechnology, as this novel technology for cell expansion can be applied to any adherent cell cultures.

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1. Introduction

The use of adequate cell sources in tissue engineering and regenerative medicine strategies is crucial for their success [1,2]. In most cases the number of cells harvested from a patient to be used in subsequent transplantation is insufficient, necessitating their expansion, while retaining their phenotype. A problem arises concerning the technologies currently available for cell expansion and proliferation [3]. Cell culture is usually performed in two-dimensional (2D) plates and enzymatic or mechanical methods are used to induce cell detachment. Besides the use of aggressive conditions for cell recovery, which might inactivate the cells, these methods of cell culture are inadequate for the production of the large numbers of cells required for research and industrial use.

This work aims to develop thermoresponsive 3D substrates, prepared using supercritical fluid techniques, as a new technology for cell expansion and proliferation. 3D thermoresponsive substrates with a large surface area allow the growth of a greater number of cells than the traditional 2D culture plates and avoid enzymatic or mechanical methods of cell recovery. Particularly interesting are thermoresponsive systems based on poly(N-isopropylacrylamide) (PNIPAAm), which are among the most widely investigated [4]. This

polymer presents, in aqueous solution, a lower critical solution temperature (LCST) of around 32 °C, which makes it extremely interesting for biomedical applications. Below the LCST it presents a flexible extended coil conformation, which makes it hydrophilic. In contrast, close to the LCST it becomes hydrophobic. The preparation of smart surfaces which can be tuned between hydrophobic and hydrophilic can either promote cell adhesion or detachment depending solely on the temperature (Scheme 1).

The preparation of the poly(D,L-lactic acid) (P_{D,L}LA) constructs was based on a supercritical fluid (SCF) technology which is well documented in the literature [5,6]. Supercritical fluids, especially supercritical carbon dioxide (scCO₂), have been identified as prime candidates to develop alternative clean processes, as they offer many advantages. CO₂ is readily available, environmentally acceptable, non-flammable, has low critical constants and leaves no toxic residue. The unique solvent tuneability of supercritical fluids, from gas-like to liquid-like properties, also offers the intriguing possibility of precise control over the processing conditions, leading to new interesting applications [7].

2. Experimental procedure

2.1. Supercritical fluid polymerization

PNIPAAm hydrogels were polymerized in a high pressure apparatus. Monomer, cross-linking agent (1.2 wt.%) and initiator

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Scheme 1. Illustration of the principle underlying the proposal in this work, thermoresponsive 3D substrates. At 37 °C the surface is hydrophobic, allowing cell attachment and growth. At lower temperatures the surface becomes hydrophilic, promoting cell detachment.

(2 wt.%) were loaded into the high pressure vessel, which was purged before the reaction was allowed to take place.

Based on the results presented by Cao et al. [8] and Temtem et al. [9], the percentage of cross-linking agent was chosen to be 1.2 wt.%, as this percentage yields 90% reaction conversion. Further, the PNIPAAm hydrogel prepared under these conditions has a LCST of 32 °C.

Polymerization was carried out, under stirring, at 65 °C and 20.0 MPa for 24 h. The operating conditions were chosen based on results described in the literature [8,9]. These experimental conditions ensure a homogeneous phase and that all the reagents are soluble in the supercritical phase.

2.2. Supercritical fluid foaming

The scaffolds were prepared by SCF foaming at 200 bar and 35 °C. In each experiment approximately 100 mg of PDLLA or a mixture PDLLA + 10 wt.% PNIPAAm was loaded into a mould, which was placed inside a high pressure vessel. The vessel was heated by means of a thin band electric heater (Ogden) connected to a temperature controller, which maintained the temperature within ± 1 °C. Carbon dioxide was pumped into the vessel using a high pressure piston pump (P-200A, Thar Technologies) until the operational pressure was attained. The pressure inside the vessel was measured with a pressure transducer. The system was closed for 30 min to allow plasticization of the polymer. Afterwards the system was slowly depressurized (~ 5 bar min^{-1}).

3. Results and discussion

The technique used to produce the 3D matrices is based on gas foaming technology, which takes advantage of the plasticizing properties of carbon dioxide. $P_{D,L}$ LA matrices were produced at 200 bar and 35 °C. A smart material can be successfully produced by incorporation of PNIPAAm into the substrate during processing. This single step procedure is of the utmost importance for the industrial production of these systems. Additionally, PNIPAAm can also be processed

using SCF technology [8,9]. In this work PNIPAAm cross-linked with *N,N*-methylenebisacrylamide was polymerized at 200 bar and 65 °C for 24 h. Penetration of the thermoresponsive hydrogel into the $P_{D,L}$ LA construct was confirmed by Fourier transform infrared spectroscopy (Fig. S1).

The morphological analysis was carried out by scanning electron microscopy (SEM) and micro-computed tomography, which allows determination of the porosity, interconnectivity and mean pore size of the materials, which were found to be 68%, 55% and 138 μm , respectively. The matrices present a rough inner structure with micro and macropores, which can encourage cell attachment and proliferation. Furthermore, the matrices are highly interconnected and thus nutrients and oxygen are accessible to the cells and cell waste can be eliminated. The pore size of the material is another important key issue, so that cells are able to penetrate the pores and then be released from the interior upon lowering the temperature.

Fig. 1 shows SEM images of the $P_{D,L}$ LA and $P_{D,L}$ LA + 10% PNIPAAm matrices colonized by L929 cells after 7 days culture. The insets represent magnified images of the materials, which show proliferation of the cells into the bulk of the matrix in more detail. Samples fixed with 4% formalin and dehydrated in a series of ethanol solutions were fractured using liquid nitrogen before being sputter coated with gold.

Fig. 1 also presents a confocal microscopic image of fibroblast-like cells after 7 days culture. Cells were stained with calcein and observed under a confocal microscope in order to evaluate the cell distribution within the construct. Green fluorescence indicates viability of the cells cultured on the matrices, and from the images we can conclude that there was good integration between the cells and the polymer.

Due to the thermoresponsive properties of the substrates, i.e. the possibility of tuning the hydrophilicity/hydrophobicity, recovery of the cells is possible simply by lowering the culturing medium temperature. The potential use of these materials in biomedical applications greatly depends on their cytotoxicity. Therefore, a cytotoxicity assessment of the matrices leachables was carried out as a preliminary approach to assessing their

Fig. 1. SEM and confocal microscope images of (a) P_{D,L}LA and (b) P_{D,L}LA loaded with 10% PNIPAAm constructs. (Insets) Cell adhesion and morphology after 7 days culture.

toxicity potential (Fig. S2). Extracts of the materials (P_{D,L}LA, P_{D,L}LA + 10% PNIPAAm and PNIPAAm) were prepared. Cells were cultured in contact with these extracts for 72 h and their viability assessed by an MTS test. Latex extracts were used as a negative control and culture medium was used as a positive control.

The matrices cultured for 1, 3 and 7 days were trypsinized and the cells recovered were counted using a haemocytometer. Trypan Blue was used in order to ensure that only viable cells were counted. Fig. 2 presents cell growth curves in terms of the relative increase in the number of cells from day 1.

The doubling time in the prepared substrates was 3 days, which is a common doubling time for cell growth in 3D structures [10].

The trends in the growth curves are similar for both substrates after 7 days culture. Fig. 3 shows the number of cells grown in the substrates after 7 days culture and the number of cells recovered after lowering the culture medium temperature. The cells detached by cooling (at 4 °C) were resuspended in fresh medium and cell detachment efficiency was determined by cell counting using a haemocytometer. The Shapiro–Wilk test was used to verify the normality of the data obtained and, as the results follow a normal

Fig. 2. Growth curves for L929 cells on the prepared matrices. Black line, P_{D,L}LA; grey line, P_{D,L}LA loaded with 10% PNIPAAm.

Fig. 3. Cell detachment efficiency of P_{D,L}LA and P_{D,L}LA loaded with 10% PNIPAAm. White bars, number of cells collected after trypsinization; grey bars, number of cells detached after cooling.

distribution, one-way ANOVA with a Bonferroni post-test was performed to compare the results for the different time points. The results were considered statistically significant at $P < 0.05$. In these experiments it was only possible to recover cells at high efficiency when the matrix is impregnated with the thermosensitive hydrogel PNIPAAm. The results represent a significant contribution to the state of the art and will further enhance tissue engineered therapeutics use in regenerative medicine, as limitations on cell expansion and proliferation may be overcome.

The detached cells were replated in a conventional cell culture plate and their viability evaluated after 72 h (Fig. S3). Observation of the cells by optical microscopy revealed the normal morphology of healthy cells, as well as showing a lower number of cells collected from the P_{D,L}LA scaffolds compared with the thermosensitive P_{D,L}LA + 10% PNIPAAm scaffolds. Furthermore, the results of the MTS test confirm the viability of the replated cells and, hence, the success of the technology developed.

4. Conclusions

A completely new strategy for cell culturing, based on the design of 3D smart surfaces which might overcome the limitations on cell expansion and proliferation of presently available scaffolds, has been developed within this work. A major advantage of this technology would be the possibility of recycling and reusing the same substrate. Nonetheless, further studies are needed to confirm this hypothesis. This technology is further envisaged to be easily scalable and it is expected to be less costly, a consequence of the possibility of continuous production and a lower labour input. We can foresee the use of bioreactors to further provide mechanical stimulus to cells cultured in such 3D environments and to incorporate bioactive agents in such smart scaffolds, including differentiation agents or growth factors. An indirect impact of this work is related to the field of biotechnology, as this novel technology for cell expansion can be applied to any adherent cell cultures.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1 and Scheme 1 are difficult to interpret in black and white. The full colour images

can be found in the on-line version, at [doi:10.1016/j.actbio.2010.09.032](https://doi.org/10.1016/j.actbio.2010.09.032).

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.actbio.2010.09.032](https://doi.org/10.1016/j.actbio.2010.09.032).

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