Energy metabolism and re-establishment of intercellular adhesion complexes of gel entrapped hepatocytes

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Abstract

We studied the effect of continuous medium flow on the viability and structural organization of hepatocytes high density entrapped in alginate gel beads in the first few hours after isolation. The metabolic energy status of the entrapped cells, monitored *in vivo* by ³¹P NMR spectroscopy, was stable during the experimental time and a physiological redox ratio was reached after the first three hours of culture. The morphological analysis revealed that the entrapped hepatocytes placed in a fixed-bed bioreactor under continuous flow showed a polyhedral shape with numerous microvilli on cell surface and reconstituted tight junctions as well as bile canalicular structures, closely resembling those present in the liver. These results suggest that continuous flow allows the culture of hepatocytes at very high cell density within a matrix without loss of viability and accelerates cellular tissue reconstruction at very short times after isolation. This type of culture could represent a very useful model for physiological and toxicological studies as well as a promising approach toward the development of a bioartificial hybrid support device in acute liver failure.

Introduction

It has been demonstrated that hepatocytes cultured in a three-dimensional matrix maintain differentiation and reorganize allowing *in vitro* reconstruction of a liver-like tissue. The re-establishment of cellular organization is of major potential interest for physiological, pharmacological and toxicological researches, as well as for the development of bioartificial organs (Berthiaume, 1996). Cell polarity and three-dimensional organization have been found to depend not only on the composition, but also on the geometry of the extracellular matrix (Ben-Ze'ev, 1988; Dunn, 1992).

Owing to their properties of biocompatibility, chemical and mechanical stability, permeability, and ability to form gels under non traumatic conditions for cells, some polysaccharides, like alginates, have been proposed as an alternative immobilization matrix

in a number of biotechnological applications (Martinsen, 1989; Shoichet, 1996). In particular, animal and human hepatocytes entrapped in alginate gel beads, coated or not, have been proposed as a possible functional component of an extracorporeal bioartificial device in liver failure (Fremond, 1993; Selden, 1998). Hepatocytes embedded in Ca-alginate beads at low cell densities (approx. 2×10^6 cells/ml) and incubated in static culture maintained most liver specific functions over a few days (Miura, 1986, 1987; Joly, 1997), and reorganized at a three dimensional level after 1–2 days of culture (Joly, 1997). However, higher cell densities may be necessary to promote cell–cell contacts and to favour cellular reorganization. This fact is of outmost importance in view of the clinical use of liver bioartificial hybrid devices requiring a hepatocyte mass that must be suitable for the metabolic support in a contained volume.

The employment of hepatocytes entrapped at high-density in matrices may result in reduced oxygen, substrates, and catabolites diffusion within the matrix, with consequent alteration of the cell metabolism and viability (Catapano, 1996; Wohlpert, 1990). In particular, oxygen supply has been shown to be critical for the adaptation of the anchorage-dependent hepatocytes (Smith, 1996) and cytoskeleton reorganization, and cell shape changes have been suggested to be energy-requiring processes (Rotem, 1992).

We have hypothesized that continuous flow with culture media of hepatocytes entrapped at high density in alginate gel beads could increase cell survival and accelerate processes of cell reorganization in the first hours after isolation.

With this aim, we have studied hepatocyte morphological-structural adaptation and changes in energy metabolism occurring during the first 8 hrs of culture under continuous flow in a fixed bed bioreactor, and we have compared the results to those obtained in static culture conditions. The metabolic study was performed using ^{31}P and ^1H Nuclear Magnetic Resonance Spectroscopy (NMR), which is known to be a powerful tool for non-invasive and non-destructive monitoring of cell physiology.

Materials and methods

Materials

Reagents, cell culture media and media components were obtained from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stated.

Isolation and culture of hepatocytes

Male Wistar rats (180–200 g b.w.), kept on a standard 'ad libitum' diet with free access to tap water, were used, according to the 'Principles of laboratory animal care'. Before surgery, the animals were anesthetized with an intraperitoneal injection of sodium thiopental (Farmotal, Farmitalia, Italy), 10 mg/100 g b.w.

Isolated cells were obtained by perfusion of the livers with collagenase (Boehringer, Mannheim, Germany), according to the method described by Moldeus et al. (Moldeus, 1978). The isolated hepatocytes were counted and suspended in RPMI 1640 (Eurobio, Bad Soden, Germany), 5% FCS (Fetal Calf Serum) (Hyclone, Logan U.S.A.), Insulin 10 μM , Hepes 15 mM and Dexamethasone 1 μM . Cell viability was assessed by the Trypan Blue exclusion test to be at least 85%.

Alginate embedding

Freshly isolated hepatocytes, suspended in complete medium, were mixed in a 0.9% NaCl solution containing 2% sodium alginate (Fluka, BioChemika, Germany) to obtain final cell densities ranging from 3.5 to 22.5×10^6 cells/ml alginate. The suspension was dropped through a syringe, equipped with a 22-gauge needle-cannula, mounted on a syringe mechanical dispenser, in a 1.1% w/v solution of CaCl_2 (pH 7.2). The beads were counted to evaluate final cell number/bead and were allowed curing for 1 min as previously described (Martinsen, 1989).

The resulting spherical beads (mean size 1.8 ± 0.2 mm) were washed in 0.9% NaCl cold solution and suspended in complete medium with 5% FCS (1 ml of medium for 2×10^6 cells). Isolation and entrapping procedures were routinely carried out within 60–80 min. The entrapped hepatocytes were placed in Petri dishes in a humidified air/ CO_2 incubator at 37°C for an hour to recover from the isolation procedure. Viability was assessed by the Trypan Blue exclusion test after dissolution of the beads in ethylene diamine-tetracetate disodium salt (EDTA) 10 mM and subsequent washes in medium.

Perfusion apparatus and experimental protocol

The medium (500 ml), placed in a water jacketed bottle and warmed at 39°C , was continuously agitated through a magnetic stirrer to reach a 37°C temperature in the bioreactor after flowing in the heat exchanger inside the magnet (Figure 1). Medium was continuously oxygenated with 95% air and 5% CO_2 at the headspace of the reservoir and by flow (1 l/min) in the external chamber of a membrane oxygenator. The homebuilt membrane oxygenator/temperature exchanger consisted of two coaxial cylinders and a loop of 2 m of silicon tubing (1 mm i.d.) (Sylastic, Dow Corning, USA) spirally rolled up around an inner glass cylinder connected to the thermostated circuit.

The membrane oxygenator was wholly built with amagnetic materials in order to position it as close as possible to the bioreactor inside the magnet for NMR experiments.

pH, pCO_2 and pO_2 were monitored off-line by sampling the medium from the bioreactor inlet and outlet by means of a gas analyzer (ABL 330, Radiometer, Copenhagen, Denmark). The aeration was sufficient to maintain the pO_2 at about 148 mm Hg, quite similar to that obtained in a humidified air/ CO_2 incubator (about 150 mm Hg). The perfusate was

Figure 1. Schematic representation of the perfusion apparatus. In A: (1) spinner vessel; (2) peristaltic pumps; (3) sampling port. In B: (4) membrane oxygenator with heat exchanger; (5) bioreactor; (6) RF coil.

recirculated in a closed manner through the bioreactor by a peristaltic pump (LKB-2115 Multiperplex, Switzerland) at 15 ml/min flow. The perfusion circuit consisted of 1 mm. i.d. luer-lock polyethylene tubing (Vygon, France) externally thermo-isolated with neoprene and, for NMR-compatible constraints, was 6 m long to and from the bioreactor. The home-made bioreactor was a cylinder with a volume of 14.5 ml and consisted of a polyethylene tube with two PVC endpieces equipped with nylon 400 μ m filters. Inside the bioreactor a glass capillar containing 20 μ l of 1 M methylenediphosphonic acid (MDPA) in Tris-HCl buffer at pH 8.9 was used as reference for NMR analysis. The bioreactor was placed vertically at the center of the magnet.

Hepatocytes, entrapped at a density of 22.5×10^6 cells/ml alginate, were loaded in the bioreactor already stabilized with fresh medium at 37 °C, pH 7.4, pO_2 148 ± 6 mm Hg, 15 ml/min flow rate. At the same time other beads were maintained in Petri dishes into the incubator at 37 °C, 5% CO_2 . Medium samples were collected at 60, 120, 180, 240,

300, 360, 420 and 480 min, at the bioreactor outlet and were subsequently analyzed by 1H NMR spectroscopy. ^{31}P NMR spectra were acquired *in vivo* from the bioreactor placed within the NMR magnet during the experimental time. At the end of perfusion, samples for ^{31}P NMR analysis of cellular extracts and for electron microscopic observation were collected.

Viability was assessed by the Trypan Blue exclusion test on entrapped hepatocytes after 8 hrs of perfusion or of static culture.

Extraction procedure

The alginate beads were extracted with the procedure previously described (Miccheli, 1988a, 1991). Briefly, the beads, washed three times with cold saline, were extracted using a 2:1 methanol/chloroform mixture and adding chloroform and water at a final 2:2:1 ratio at 4 °C. The beads were homogenized in a PCU potter (Kinematica GmbH, Kriens, Luzern, Switzerland) and centrifuged at $10\,000 \times g$, 20 min, at 4 °C. The resulting two phases were separated, dried under N_2 flow

and maintained at -80°C before being analyzed. Only the data from the aqueous phase are discussed here.

NMR spectroscopy

In vivo NMR experiments were performed on a SMIS IVS System (England) operating at 4.7 T (Tesla) equipped with a horizontal magnet, 14 cm bore. In order to achieve the best sensitivity, a home-made volume RF (Radio Frequency) coil fitting exactly to the bioreactor was built (coil i.d. 28 mm). The coil was originally tuned to the proton resonance frequency, while the phosphorus frequency was reached without removing the coil from its place by adding the proper capacitance in parallel between the coil and the resonant circuit. *In vivo* ^{31}P -NMR spectra were obtained at 81.5 MHz on the SMIS Spectrometer collecting 2048 data points on a 12.5 KHz spectra width after a 90° pulse (100 msec) and then waiting for 3 sec to allow T_1 relaxation to occur. 2048 scans were collected using CYCLOPS phase cycle. A capillar containing a known amount of methylenediphosphonic acid was fitted inside the bioreactor to allow phosphorus-containing metabolites to be quantitatively analyzed.

For ^{31}P NMR spectroscopy on extracts, samples were resuspended in 0.4 ml D_2O in a 5 mm tube with a coaxial capillar containing 50 μl of methylendiphosphonic acid (MDPA) 44 mM (pH 8.7) and were analyzed on a Varian XL 300 spectrometer (USA) operating at 121.4 MHz. ^{31}P spectra were obtained by using a 45° radiofrequency pulse, with an interpulse delay of 3 sec, 10 000 Hz spectral width, 16 Kwords.

For ^1H NMR spectroscopy, 0.4 ml of medium were added to 0.1 ml of trimethylsilylsodiumpropionate (TSP) 10 mM (Wilma, USA) in D_2O . High resolution ^1H NMR spectra were obtained on a Bruker AM 500 spectrometer operating at 500.1 MHz using a 45° pulse, 6000 Hz spectral width and 64 Kwords. The acquisition time was 5.44 sec and an additional delay of 10 sec was applied. Sixty-four scans were accumulated for all the spectra and the residual water signal was eliminated by a standard solvent suppression sequence included in the NMR spectrometer library. NMR data were processed off-line using WIN-NMR software (Bruker-Franzen Analytik GmbH, Bremen, Germany). Resonance assignments were made by comparing to data reported in the literature (Sze, 1990) and by adding small amounts of pure compounds to the extracts. Peak areas of signals at 1.33 and 2.38 ppm were used to quantify lactate and pyruvate, respectively.

Transmission electron microscopy

Immediately after withdrawal from the bioreactor, or after culturing in the standard incubator, calcium-alginate gel beads containing hepatocytes were fixed with 2.5% glutaraldehyde in 0.1 M cacodilate buffer, overnight at 4°C . Samples were then washed in 0.1 M cacodilate buffer and dehydrated in a graded series of ethanol and embedded in Spurr resin.

Ultrathin sections were obtained using a Reichert Ultracut ultramicrotome, stained with uranyl acetate and lead citrate. Examination was performed under a Philips CM12 electron microscope.

Statistical analysis

t-Student's test and a non-parametric U-test of Mann-Whitney was applied for significant variations; significance was accepted at $p < 0.05$.

Results

Viability of hepatocytes entrapped in alginate gel beads

The viability of freshly isolated rat hepatocytes was not modified by the alginate entrapment procedure, as evaluated by the Trypan Blue exclusion test on freshly prepared beads containing hepatocytes at a density $\sim 2 \times 10^6$ cells/ml alginate.

In static culture conditions, the loading of the beads with increasing amounts of cells caused a density-dependent decrease of viability after 1 hr incubation. The viability was $91 \pm 3\%$, $76 \pm 6\%$ and $69 \pm 3\%$ at $3.5 - 13.5 -$ and 22.5×10^6 cells/ml alginate, respectively.

Following 8 hrs of culture within a continuous flow bioreactor at the highest density used (22.5×10^6 cells/ml alginate), the initial viability had decreased only by $7 \pm 4\%$ while it further decreased of another 50% in static culture.

In vivo ^{31}P NMR spectroscopy on bioreactor

In Figure 2 are reported *in vivo* ^{31}P NMR spectra acquired after 1 hr (A) and 8 hrs (B) from the beginning of perfusion of the alginate beads loaded at 22.5×10^6 cells/ml alginate within the bioreactor. The resonances from high-energy phosphates are relative to α -, β -, and γ -nucleotide triphosphates (NTP), with the majority of signals being due to adenosine-triphosphate

Figure 2. ^{31}P NMR spectra obtained on hepatocytes entrapped at 22.5×10^6 cells/ml alginate after 1 hr (A) and 8 hrs (B) of perfusion under flow condition in the bioreactor.

(ATP). To the α -NTP signal contributed also the resonances of diphosphodiester (DPDE), mainly nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Phosphodiester (PDE) resonances are mainly relative to glycerophosphorylcholine (GPC), glycerophosphorylethanolamine (GPE) and other phosphodiester, such as glycerophosphorylglycerol (GPG) and glycerophosphorylinositol (GPI). Inorganic phosphate signal is mainly due to P_i in the flowing perfusate. Phosphomonoesters (PME) are partially overlapped by the P_i signal. A slight decrease in NTP levels, evaluated by $(\text{NTP}/\text{MDPA})_{\text{final}}/(\text{NTP}/\text{MDPA})_{\text{basal}}$ ratio (0.91 ± 0.03), was observed after 8 hrs of perfusion, and a significant decrease in the PDE signal was concomitantly observed (Figure 2).

^1H NMR spectroscopy on perfusate and ^{31}P NMR spectroscopy on cellular extracts

An increase in pyruvate level measured in the perfusion medium, as a function of time, was evident in ^1H NMR spectra (Figure 3), while a decrease in the lactate/pyruvate ratio was observed during the first 3 hrs of perfusion (Figure 4), then to remain constant.

Figure 3. Expanded regions of ^1H NMR spectra of medium showing the increase in pyruvate signal as a function of perfusion time. 0 time ^1H NMR spectrum corresponds to RPMI 1640 medium before cell loading of the bioreactor.

Table 1 reports the results relevant to some intracellular phosphorylated metabolites evaluated by ^{31}P NMR spectroscopy on cellular extracts obtained after 8 hrs

Figure 4. Lactate/pyruvate ratio in medium obtained from ^1H NMR data as a function of perfusion time. The results are expressed as mean $\pm$ S.D. of three independent experiments.

Table 1. Phosphorylated metabolite concentrations as evaluated by ^{31}P NMR spectroscopy on hepatocyte extracts

Metabolites	Cell static culture	Bioreactor with flow
αGP	1.14 ± 0.78	$0.29 \pm 0.10^*$
PCho	1.30 ± 0.36	$0.27 \pm 0.10^*$
GPE	0.46 ± 0.07	$0.20 \pm 0.10^*$
GPC	1.24 ± 0.08	$0.56 \pm 0.26^*$
DPDE ₁	1.29 ± 0.05	1.56 ± 0.37
NTP	1.20 ± 0.23	$1.98 \pm 0.06^*$
DPDE ₂	0.29 ± 0.16	0.62 ± 0.26

Values are means $\pm$ S.D. of three independent experiments and are expressed as $\mu\text{mol}/10^8$ viable cells.

DPDE₁ are mainly represented by NAD, NADPH, CDP-Choline, CoA; DPDE₂ are mainly represented by UDP-Glc, UDP-Gal, etc.

* $p < 0.05$.

of static culture and of perfusion within the bioreactor. The metabolite concentrations were normalized to 10^8 viable cells, taking into account the final cell mortality as measured by the Trypan Blue exclusion test.

Hepatocytes perfused in the bioreactor showed higher intracellular NTP and diphosphodiester levels (DPDE₂), mainly UDP-glucose and UDP-glucuronic

acid (Cohen, 1987; Malloy, 1986), than the static culture-maintained hepatocytes.

Furthermore, the hepatocytes entrapped in alginate gel beads and maintained in incubator showed an increase in the level of phospholipid metabolites, namely phosphorylcholine (PCho), glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE), compared to the bioreactor perfused ones. An increase of the α -glycerophosphate (αGP) levels was also observed.

Morphological analysis

Light microscopy showed that the immobilization of rat hepatocytes into alginate beads at a density of 22.5×10^6 cells/ml alginate produced no significant cell alteration, as the viable hepatocytes appeared morphologically intact (Figure 5a). Hepatocytes, initially present as isolated cells, tend to aggregate forming small groups; at 8 hrs of perfusion, they acquired a typical polyhedral shape and reorganized into a three-dimensional configuration resembling trabeculae-like structures, as shown by phase contrast microscopy (Figure 5b).

Figure 5. Light microscopy photomicrographs of rat hepatocytes entrapped into alginate gel beads. a) Section of entrapped hepatocytes, stained with hematoxylin and eosin, showing that hepatocytes were not damaged by encapsulation into alginate gel beads; b) phase-contrast light photomicrograph of hepatocytes cultured in the bioreactor for 8 hours; in this condition hepatocytes form aggregates and reorganize into a three-dimensional configuration. Magnifications: (a) 750 $\times$, (b) 500 $\times$.

Transmission electron microscopy reveals that the entrapped hepatocytes cultured in the bioreactor display a well preserved ultrastructural morphology (Figure 6a); numerous mitochondria filled the cytoplasm, and normally developed rough endoplasmic reticulum and Golgi complexes were also found. Interdigitating processes between neighboring cells were seen and, interestingly, most of contact areas consisted of completely re-established cell–cell junctional complexes and reconstituted bile canaliculi-like structures with typical microvilli facing the lumen (Figure 6c). On the contrary, plasma membranes of entrapped hepatocytes maintained in the incubator appear completely devoid of microvilli and in the regions between neighboring cells, cell–cell junctions and bile canaliculi structures were never observed (Figures 6b,d).

Discussion

A proper three-dimensional cytoarchitecture of hepatocytes in culture has been suggested to be fundamental to maintain metabolic differentiated functions like urea and albumin synthesis, as well as cytochrome P-450 activity-dependent metabolism (Berthiaume, 1996; Parsons-Wingerter, 1993). The re-establishment of a tissue-like cell organization has been obtained in culture, modifying the composition or geometry of the extracellular matrix, or the medium components, or favouring cell–cell contacts in cellular aggregates or spheroids (Moghe, 1996; Dunn, 1992; Landry, 1985; Peshwa, 1996; Falasca, 1998). Hepatocytes have been demonstrated to be able to restore cell–cell contacts and to form bile canaliculi domains, thereby maintaining a differentiated liver function, when entrapped in matrices constituted by alginate gel. Cells were, however, dispersed at a low density and their reaggregation was observed only a few days after entrapping (Joly, 1997; Guyomard, 1996; Selden, 1998; Elcin, 1998). A higher cell density may accelerate the morphological-structural reorganization, provided that an adequate mass transfer of O₂, substrates, and catabolites is secured.

Our results show the effect of perfusion in viability, energy metabolism and morphological-structural organization over the first few hours of culture of hepatocytes entrapped in alginate gel beads at high density in a fixed-bed bioreactor. The cell ATP levels measured through ³¹P NMR at the beginning and at the end of perfusion remained unchanged, indicating that energy metabolism was stable and cell viability maintained.

Figure 6. Transmission electron micrographs showing ultrastructural morphology of hepatocytes entrapped into alginate gel beads and cultured at 37 °C for 8 hr in the bioreactor (a,c) or in a standard incubator (b,d). (a) Hepatocytes cultured in bioreactor are aggregated in small groups, cell-cell junctional complexes appear to be re-established (arrows) and even bile canaliculi-like spaces are present (*). These cells also display a well preserved morphology as for nucleus and cytoplasmic organelles. Finally, note the presence of regular microvilli on cell surface. (b) Electron micrograph of hepatocytes cultured in a standard way. In this condition, in the region of plasma membrane between neighboring hepatocytes cell-cell adhesion areas are not reconstituted and cell surfaces appear completely devoided of microvilli. (c) Higher magnification of rebuilt cell-cell contracts between two hepatocytes cultured into the bioreactor. Reconstituted bile canaliculi-like structures (bc), with typical microvilli, and tight junctions (arrows) are clearly evident. (d) Detail of the plasma membranes of neighboring hepatocytes cultured in a standard incubator. Cell surfaces were never seen to interdigitate, nor cell junctions were found (arrow heads). Magnifications: (a,b) 5700 $\times$; (c,d) 32 000 $\times$.

Furthermore, the stability of the lactate/pyruvate ratio in the perfusion medium, reflecting the cytosol NADH/NAD⁺ ratio (Gudbjarnason, 1962), demonstrated that a physiological redox state was attained following the first 3 hrs after the onset of perfusion.

Polyhedral shape, microvilli, tight-junctions and bile canalicular structures were reformed after 8 hrs of perfusion within the bioreactor, showing that such a short time was sufficient to restore a liver-like ultrastructural intercellular organization.

In agreement with previous results (Hua, 1993; Kan, 1998; Pollok, 1998; Wu, 1996), our findings show that an adequate oxygen and substrate supply is critical for the molecular processes occurring during cell reorganization, although the exact nature of these events requiring metabolic energy is still unclear. In fact, in hepatocytes entrapped at the same density but cultured under static conditions, neither specialized adhesion structure nor surface microvilli were present. Hepatocytes in static culture also exhibited significantly lower levels of NTP and DPDE (like UDP-glucose), and higher levels of glycerol-3-phosphate, which reflect cytosolic NADH/NAD⁺ ratios (Ben-Yoseph, 1993), than the hepatocytes under continuous flow conditions. These changes in the cytosol redox state may be related to an inhomogeneous oxygen supply within gel beads, in analogy to findings in aggregates or spheroids (Kurosawa, 1989).

A hypoxia-dependent injury of membranes may be suggested by the increase observed in GPE levels in static culture. Phosphodiester (GPC, GPE) levels may reflect the deacylation-reacylation process of membrane phospholipids (Morash, 1989), which is related to the cell energy status (Miccheli, 1988a). Under hypoxia conditions, the ATP-dependent reacylation process is reduced, and the enhanced phospholipid hydrolysis, which is catalyzed by phospholipase A₂ and lysophospholipase, leads to the production of lysophospholipids and phosphodiesters, respectively (Miccheli, 1988b).

The decrease in phosphodiester levels observed in the bioreactor during perfusion may be consistent with the regulation of membrane phospholipid metabolism related to the continuous flow-dependent improvement of cell energy balance.

In conclusion, the continuous flow in a bioreactor containing hepatocytes entrapped at high density in alginate gel beads accelerates the processes leading to three dimensional cellular re-organization, reproducing tissue-like cell structure. Since alginate is a very poor attachment substrate, the reaggregation process

in gel matrix could occur as a consequence of high cell density. Cell-cell contacts could be re-established through a mechanism mediated by membrane extensions (Powers, 1996) or by preventing cells from spreading (Mooney, 1995). The cell structure reorganization concerning membrane domains is likely dependent on the energy state.

The system we have described here may represent a novel liver-like culture model to be used in pharmacological and toxicological studies. Finally, because cell entrapping in alginate gel beads has been shown to be suitable to increasing cell viability and function in hypothermia and cryo-preservation (Guyomard, 1996; Selden, 1998), the preconditioning under continuous flow, that we propose, may be a first step for the preservation of organized and functional cells, readily available on demand. Upon appropriate embedding, these cell/matrix constructs could be used both for cell heterotopic transplantation and as extracorporeal bioartificial liver assist devices.

The use of NMR techniques as applied in this study represents a largely applicable method for the evaluation of other polymer/mammalian cell constructs designed for different biotechnological applications.

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