Microenvironment-dependent respiration of T-47D cells cultured in alginate biostructures

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Abstract

Objectives: The main objective of this paper was to investigate whether the oxygen consumption rate (OCR) of cells entrapped in alginate hydrogels depends on presence of soluble factors present in foetal bovine serum (FBS).

Materials and methods: Pericellular oxygen concentrations were measured using a photochemical oxygen sensor inserted into bioconstructs made from different formulations of alginate, containing T-47D cells. Cell count was corrected for viability as determined by cell uptake and exclusion of standard live/dead fluorophores, in sections of freshly prepared biostructures. Based on concentration data, OCR of the embedded cells was calculated according to a simple algorithm.

Results: OCR was found to vary significantly between the different formulations investigated. Inclusion of high concentrations of FBS in the biostructure matrices elicited significantly higher OCRs, in guluronate-rich gels similar to those previously found in monolayer culture. Guluronate-rich gels also generally permitted highest OCR. Respiration also had a falling tendency with increasing alginate concentration and elastic modulus.

Conclusions: Presence of FBS in biostructure matrices elicited higher OCR in T-47D cells. Formulation of biostructures must consider differential diffusion of macromolecular substances.

Introduction

The field of 3D cell cultures is fast expanding, owing to its promises in areas such as regenerative medicine and cancer modelling. The ability of cells to thrive in 3D culture is, however, strictly limited by availability of nutrients. Any structure exceeding a few hundred micrometres is challenged by lack of oxygenation when employing cell densities approaching those of native tissues (1). To design viable tissue surrogates that balance diffusion paths, cell density and baseline nutrient demands, it is thus necessary to characterize the specific devices.

Although use of allogeneic lysates is an option (2), mammalian cell cultures still rely heavily on poorly defined xenogeneic sera. This is a consequence both of a need to better understand the complex signalling environments that natural tissues experience, and costs incurred when attempting to formulate defined media of sufficient complexity (3).

To allow cells in devices intended for implantation to establish cell communication with the native signalling environment, it is necessary to allow passage of large macromolecular substances through the gel networks of the immobilization matrix. The diffusive and perfusive capacities of a specific device would, among other factors, depend on the matrix material being used (4). Some important molecules, such as insulin, diffuse readily through most gels used. Larger proteins may have their diffusion impaired by the gel network (5). Gel pore size and tortuosity are thus important formulation aspects when matrices for tissue-emulating devices are designed.

Alginate is a copolymer of mannuronic and guluronic acids, obtained from bacterial or algal sources (6). The ratio of monomers, their pattern of distribution within a given chain, the occurrence and length of homomeric or strictly alternating blocks, all depend on a sample's origin. Even alginates extracted from the same

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species of alga vary significantly in composition, depending on which structure (e.g. stipes, leaves) they have been extracted from. These factors play a major role in determining the final gel structure (6). Commercially available alginates are commonly defined by their viscosity, which does not provide sufficient information for defining the gel's inner structure, when it is used cell immobilization.

Calcium alginate hydrogel is a promising matrix for cell immobilization in regenerative medicine. It gels under benign conditions, at physiologically relevant concentrations of Ca^{2+} , allowing high proportions of viable cells to be directly immobilized within, and has tailorable viscoelastic properties (6,7). It has been shown to be widely biocompatible (8,9). Although neither humans nor common animal model organisms possess specific biodegradative enzymes for alginate, it is possible to formulate gels for injection or implantation, that display a range of degradation properties (10).

Calcium alginate-based systems are currently being used in a number of clinical applications as established medical products, or are undergoing clinical trials such as for treatment of diabetes, myocardial infarction, haematoma, vesicourethral reflux problems, cartilage regeneration and use in lung cancer surgery (11–15). However, it is a common occurrence in the literature that key parameters for the characterization of devices used are omitted, among which are (a) monomeric composition, (b) molecular weight and (c) degree of calcium saturation of alginates used to form the investigated bioconstructs. To compare results from experiments using calcium alginate as a matrix, it may be necessary to establish a greater degree of standardization in the characterization of the calcium alginate used. This might hold true for other matrix materials also.

Previous research has suggested that alginate matrix composition may influence oxygen consumption rates (OCRs) of entrapped cells (16), and it has long been known that monomeric composition of alginates influences the diffusion rates of large soluble proteins immobilized in gels formed from them. (17). The existence of anchorage motifs in an immobilization matrix, and its viscoelastic properties, have also been shown to influence the differentiation and metabolism of immobilized cells. (18). In a previous work, our group presented a model that allows characterization of oxygen metabolism of cells entrapped in alginate 3D structures (19). Here, we have used this model to determine how matrix composition influences OCRs of cells immobilized in different matrices, with known physical characteristics, in the presence and absence of foetal bovine serum (FBS).

Materials and methods

Cell culture and extraction

Human T-47D cells (20), a line derived from a mammary carcinoma (which exhibits properties of epithelial cells), were cultured in 25 or 125 cm² standard cell culture flasks, and kept in continuous exponential growth by reculturing 2–3 times per week, as previously described (21). Medium used was standard RPMI 1640, supplemented with 130 IU/l human recombinant insulin (Sigma, St. Louis, MO, USA), 1% penicillin–streptomycin (Euroclone, Pero MI, Italy, art. no. ECB3055D) and 10% FBS (Euroclone, art. no. ECS0180L). Reculturing and harvesting was performed by removing growth medium, rinsing three times in 1.5 ml trypsin solution and incubating the culture for 5 min at 37 °C in the trypsin solution remaining after aspiration. The cells were resuspended in medium and the number of extracted cells was estimated by counting 10 pre-determined plots using a haemocytometer. Cell suspension to be added to the alginate system was drawn from the culture flask using a micropipette, after flushing the suspension through the pipette several times to achieve a homogeneous mixture. Work with cell cultures was performed in laminar air flow benches using aseptic work techniques.

Alginate biostructures

Alginate gels were prepared on the basis of sodium alginate solution and a dispersion of calcium alginate particles that initiate gelling upon mixing (22). All alginate materials were obtained from FMC Biopolymers/NovaMatrix (Sandvika, Norway). Four main types of formulation were used.

First, simple formulations based on 2% w/w solutions of alginate were prepared. For these, sodium alginate component was prepared by adding 12.25 ml sterile filtrated aqueous 4.6% D-mannitol (Sigma) to pre-sterilized vials of freeze-dried alginate, containing 250 mg dry material. This was of either low chain weight mannuronate-rich ($F_G = 0.44$) sodium alginate (PRONOVA SLM-20, batch no. 209020) or low chain weight guluronate-rich ($F_G = 0.67$) sodium alginate (PRONOVA SLG-20 batch no. 221105). For this formulation, calcium alginate (PRONOVA Ca M, $F_G = 0.46$ batch nos. 701-05, 703-05 and BP-1109-23) was likewise mixed with 4.6% mannitol solution to 2% w/w suspension which was sterilized by autoclaving at 121 °C for 30 min. Particle size of the calcium alginate particles, as stated by the manufacturer, was in the range of 45–87 nm, and calcium content 9.6–10.2%.

Formulations containing high concentrations of FBS were prepared by adding 8.33 ml FBS (Sigma-Aldrich, lot no. 089K3395) containing 1% penicillin–streptomycin (10 000 U/ml, 10 000 µg/ml respectively; Biochrom AG, Berlin, Germany) to 250 mg vials freeze-dried sodium alginate, to obtain 3% w/v solution. For this purpose, PRONOVA SLM-20, batch no 111105/1 and PRONONVA SLG-20 batch nos. 130707/1-2 were used, mannuronate-rich and guluronate-rich respectively. To accommodate high concentration of FBS in the alginate matrix, it was necessary to use a different calcium donor. Calcium is released from calcium alginate in an ion-exchange reaction, and presence of counter ions in saline solutions such as FBS induces a very rapid release of calcium from calcium alginate particles. In the present set-up, this would have led to gels setting within the syringes and losing much of their mechanical strength before being dispensed. To control speed of gelation, short-chained Ca alginate oligomer (Ca oligo-G, batch no. BU-1107-01) with slower gelling kinetics was chosen to be dispersed in 4.6% aqueous D-mannitol solution at concentration 2.5% w/v.

Formulations containing a balanced salt solution were prepared by adding 8.33 ml Hank's balanced salts (Sigma) to vials containing 250 mg of freeze-dried sodium alginate (SLM-20, batch no 111105/1 and PRONONVA SLG-20 batch no. 130707/1, mannuronate-rich and guluronate-rich respectively). 2.5% w/v dispersion of slow-gelling, short-chained alginate oligomer (Ca oligo-G, batch no. BU-1107-01) was prepared in 4.6% aqueous D-mannitol solution.

To enable direct comparison with unmodified gel, similar formulations with sodium alginate dissolved in 4.6% D-mannitol were also prepared, using the same

materials. For full overview of the formulations used, consult Table 1.

Alginate solution and calcium alginate dispersion were dispensed in two syringes joined by a three-way connector. Exactly, 4.0×10^5 cells suspended in 100 µl of growth medium were added to calcium alginate dispersion by carefully emptying the micropipette at the nozzle of the syringe while retracting its piston. The two gelling components were transferred through the connector between syringes 10 times in a rehearsed manner to initialize gelling. Contents were emptied into standard test tubes (acid-cleaned borosilicate glass; Pyrex, Lowell, MA, USA) 75 mm in length, and internal diameter of 10 mm, through the three-way connector. The biostructure was left for 5 min to allow initial gelling to occur, and subsequently covered with 2.5 ml RPMI 1640 medium.

Cell viability assessment

500 µm vibratome (Microm HM 650V, Thermo Scientific Microm, Waldorf, Germany) sections of biostructures (prepared during the same general time frame as oxygen measurement experiments, using the same batches of sodium and calcium alginates and the same cultures of T-47D cells) were taken approximately 2 h after seeding, to assess numbers of viable cells. Sections were cut using blade speed 0.5 mm/s, amplitude 1.2 mm and blade oscillation frequency 70 Hz. Sections were then incubated with calcein and propidium iodide (Fluka Biochemika art no. 04511, Sigma-Aldrich, St. Louis, MO) or calcein AM and ethidium homodimer (Molecular Probes, L-3224, Eugene, OR) in Hank's balanced salts (Sigma-Aldrich, H2387-10XK, St. Louis, MO), for

Table 1. Descriptions of all gel formulations used and their abbreviations

Formulation name	Sodium alginate (soluble)	Sodium alginate solvent	Calcium alginate (water insoluble calcium donor)
M-0-M	SLM-20 (~45% guluronate)	4.6% aqueous D-mannitol	PRONOVA CaM (~45% guluronate)
M-0-G	SLM-20 (~45% guluronate)	4.6% aqueous D-mannitol	Calcium oligo-G (>90% guluronate)
M-H-G	SLM-20 (~45% guluronate)	Hanks' balanced salts solution	Calcium oligo-G (>90% guluronate)
M-S-G	SLM-20 (~45% guluronate)	FBS	Calcium oligo-G (>90% guluronate)
G-0-M	SLG-20 (~70% guluronate)	4.6% aqueous D-mannitol	PRONOVA CaM (~45% guluronate)
G-0-G	SLG-20 (~70% guluronate)	4.6% aqueous D-mannitol	Calcium oligo-G (>90% guluronate)
G-H-G	SLG-20 (~70% guluronate)	Hank's balanced salts solution	Calcium oligo-G (>90% guluronate)
G-S-G	SLG-20 (~70% guluronate)	FBS	Calcium oligo-G (>90% guluronate)
G/M-H-G	1:1 SLG-20 and SLM-20	Hanks' balanced salts solution	Calcium oligo-G (>90% guluronate)

The formulation abbreviations are on the form <soluble alginate type>-<alginate solvent>-<calcium alginate type>. Two types of soluble alginates were used, with similar molecular weights (~20 000 kDa) but different guluronate content, mannuronate-rich (M) and guluronate-rich (G). High guluronate content increases the number of interactions per cross-linking site. Three solvents were used: 4.6% (w/w) D-mannitol in water (O), FBS (S) and Hanks' balanced salt solution (H). Two types of insoluble calcium alginate particles were used as calcium donors: One based on unmodified alginate chains having a rapid calcium release profile (PRONOVA CaM, M) and one based on all-guluronate alginate fragments, having a higher affinity for calcium and thus a slower release profile (G). Several different solvents were used for the sodium alginate component, whereas calcium alginate particles were always dispersed in 4.6% aqueous D-mannitol.

15 min at 37 °C in standard 35 mm Petri dishes (please see Fig. 3 for details on which fluorophores were used in specific experiments).

Confocal microscopy (Nikon, Düsseldorf, Germany) image stacks were recorded with excitation laser light at 488 and 543 nm, and measuring emission light at 515–530 nm (calcein, viable cells) and 605–675 nm (propidium iodide/ethidium homodimer, dead cells). An inverted microscope was used, with objective magnification at 10×. Controlling software alternated pairs of excitation and detection frequencies to exclude cross-talk, and stored the average of a number of scans sufficient to achieve a noise level of at most 14 dB. Images were stacked in .ics/.ids format files by the imaging software, loaded into ImageJ (National Institutes of Health, Bethesda, MD, USA), and analysed using a plugin (3D Object Counter; Fabrice P. Cordelières, Institut Curie, Orsay (France)). A custom Matlab (The Mathworks, Natick, MA, USA) script was used to find double-stained cells, which were counted as non-viable. Although nominally cut to a thickness of 500 µm, low gel strength led to deformations during cutting. Depth of sections assessed was typically ~300 µm, yielding a stack of ~60 sections.

Oxygen measurement and determination of oxygen consumption rate

Test tubes with medium-covered biostructures were transferred to a pre-sterilized Forma Scientific (Marietta, OH, USA) incubator (model 3862) in HEPA-filtered atmospheric air with 5% CO₂ and 80–85% relative humidity. A syringe-mounted needle-type fibre-optic oxygen microsensor with flat-broken tip of 140 µm diameter (PreSens GmbH, Regensburg, Germany) was used with a PreSens Microx TX3 oxygen meter, for continuous logging of oxygen concentration at a predefined depth inside the biostructures. Calibration was performed against 3% NaSO₃ (Fluka/Sigma Aldrich) (0 ± 0.1% O₂) and water vapour saturated ambient air (21.0 ± 0.1% O₂), using current meteorological data to account for barometric pressure.

For each oxygen concentration measurement series, the continuous derivative function was calculated using Origin 8.5 (OriginLab, Northampton, MA, USA). Oxygen consumption rate was found by correcting resulting values for cell density according to eqn (1).

$$R = \frac{\Delta n}{\Delta t \cdot N_m} = \frac{(C_1 - C_2) \cdot V_m}{(t_1 - t_2) d_c V_m} \quad (1)$$

R is OCR per cell (mol/cell/h). Δn and Δt are changes in the amount of oxygen (mol) in time (h), N_m is

number of metabolizing cells (unitless). C_1 and C_2 are oxygen concentrations (mol/l) measured at t_1 and t_2 (h), respectively, and V_m is the volume of the biostructure in litres – although, as can be seen from the equation, this term is cancelled out. d_c is the density of viable cells in the gel (cells/l). Subscripts 1 and 2 denote separate measurement times. The density of viable cells was calculated according to eqn (2). The point where oxygen concentration measurements were taken was located 8–9 mm from the biostructure–growth medium interface, as shown in Figure 1. The sensor was placed by directly observing that the photochemically active filament was vertically aligned with the point where the test tube began to curve inwards towards the bottom.

$$d_c = \frac{S \cdot N_0}{V_{gel}} \quad (2)$$

Here S denotes viability estimated as described in the previous section. N_0 denotes the number of cells originally added to the gel as estimated by counting samples of cell suspension in five plots of two separate Bürker chambers, and V_{gel} is the volume of hydrogel used. This procedure was described in greater detail in our previous work (19).

Oscillatory rheological measurements

Rheological measurements were performed on a Physica MCR 300 oscillatory rheometer (Anton Paar, Graz,

Figure 1. Photograph of the sensor embedded in the gel (a) and basic schematic (b) of the experimental set-up used for oxygen measurements. The dimensions of the test tube holding the biostructures were 75 mm (length) × 10 mm (internal diameter). The biostructure filled the lower 13 mm of the test tube, including the rounded bottom. The height of the growth medium column, measured from the gel-medium interface, was approximately 24 mm.

Austria) (Measuring system: PP50/P2, serrated probe, gap: 1 mm, frequency: 1 Hz and strain: 0.005). Self-gel kits, as described above, were mixed in a timed and standardized procedure, and contents were emptied on to the sample holder. Data were recorded for 60 min, and fitted to eqn (3).

$$G' = A(1 - f \cdot e^{-k_1 t} - (1 - f)e^{-k_2 t}) \quad (3)$$

Here, G' is the elastic modulus, A the final gel elastic modulus, f a distribution parameter, and k_1 and k_2 are kinetic constants characteristic of the gel formulation (unpublished data, not shown).

Software and statistics

Origin 8.5 (OriginLab, Northampton, MA, USA) and Excel 2010 (Microsoft, Seattle, WA, USA) were employed for all statistical analyses, using either built-in functions or algorithms derived from standard text books. All estimates are given as mean $\pm$ standard error, unless otherwise stated, and are based on at least three independent experiments. Comparisons of respiration rates in Figure 3 were performed using one-way ANOVA and *post hoc* Tukey's honest difference test. Remaining statistical analyses were performed using Student's *t*-tests and an unmodified threshold for statistical significance at $P < 0.05$. Grubb's *G*-test was used in a single analysis for outliers. Tests of whether linear regression slopes differed significantly from 0 were performed using built-in algorithms in Origin 8.5, using the instrumental weighting method in the case of data in Figure 7. Testing concerning differences between the two slopes in Figure 5 was performed using an Excel 2010 spreadsheet and test statistic calculated using eqn (4). Here, b_1 and b_2 are the regression slopes, while $s_{b_1-b_2}$ is the pooled standard error, calculated according to (5).

$$t = \frac{b_1 - b_2}{s_{b_1-b_2}} \quad (4)$$

$$s_{b_1-b_2} = \sqrt{\frac{(n_1 - 2)s_{res,1}^2 + (n_2 - 2)s_{res,2}^2}{(n_1 - 2) + (n_2 - 2)}} \sqrt{\frac{1}{s_{x,1}^2(n_1 - 1)} + \frac{1}{s_{x,2}^2(n_2 - 1)}} \quad (5)$$

s_{res}^2 denotes the variance of regression residuals, s_x^2 the *x*-variance and indices 1 and 2 refer to the two data sets analysed in the regression procedures.

Results

Viability

Several alginate hydrogel formulations were designed to assess possible differences between metabolic rates in guluronate-rich and mannuronate-rich gels. To assess viability of cells entrapped within biostructures composed of these gels, three samples from each formulation were analysed approximately 2 h after preparation. Viability of entrapped cells was generally 85–90%, although some guluronate-rich samples had slightly lower values. Viability ratios registered for each formulation were therefore used for calculation of OCRs for cells immobilized in biostructures made from the respective formulation.

Oxygen concentration measurements

Oxygen concentration recordings using the photochemical sensor were plotted as a function of time. Initially, most curves appeared to have a slowly diminishing rate of reduction in oxygen concentration, but later, especially if oxygen persisted within the system for more than about 10 h, some showed an increasing tendency (Fig. 2). All samples depleted initial oxygen stores to the point where oxygen concentration fell below the sensor's sensitivity threshold within 40 h, but only measurements

Figure 2. Oxygen concentration time series obtained when measuring in biostructures prepared using the formulations G-0 (dotted lines) and M-0 (full-drawn lines). Each line represents a measurement series performed in a distinct biostructure prepared separately. Oxygen concentrations above approximately 205 μM are artefacts owing to temperature and CO_2 equilibration. Red lines and indices illustrate schematically how data were used in order to calculate the OCR according to equation 1 (also overlaid on the figure). The difference was calculated over 6–12 time increments beginning at 2.5 h into the experiment and ending at 5 h. The resulting values were averaged to one estimate for that time series, and in all cases the average was based on at least three parallels.

recorded within 2.5 to 5 h into the experiments were used to calculate OCRs. The measurement series recorded in biostructures composed of guluronate-rich gel containing high concentration of FBS is an exception: in this formulation, oxygen concentration fell so quickly that it was necessary to use an earlier and shorter time frame, from 0.5 to 2 h into the experiment. For the first samples examined (G-0-M and M-0-M), calculation interval between measurements was set to 30 min, but to obtain better resolution, it was later decided to reduce this to 5–10 min (all other formulations). The increased number of data points, however, had minimal impact on R^2 values of linear regressions. As described previously, there was no significant fall in viability during the first 24 h in biostructures cultured under similar conditions (19), a finding we have also confirmed for several matrices and other types of cells (unpublished data).

There were evident differences between oxygen concentration time series recorded in guluronate-rich and mannuronate-rich gels of otherwise equal composition (see Fig. 3). For all G/M formulation pairs, except that

Figure 3. Viability and oxygen consumption rate (OCR) of cells 2.5–5 hours after being immobilized in biostructures made using different alginate gel formulations (Table 1). Both white and black bars begin at 0. Viability values represent the fraction of cells staining with calcein or calcein-AM only. Propidium iodide was used as stain for non-viable cells for G-0-M and M-0-M. Ethidium homodimer was used for all other formulations. There are no statistically significant differences between the viability values registered. The mean OCR of cells immobilized in the various matrices were calculated based on oxygen concentration measurements taken at a point 8–9 mm within the biostructures using a photochemical oxygen sensor. There is an overall tendency that respiration is higher in guluronate-rich gels, and where foetal bovine serum is present. All differences statistically significant at the $p < 0.05$ level between formulations are linked by significance bars with asterisks, except the statistically significant difference between all formulations beginning with M and all beginning with G ($p = 0.03$, $df = 24$. M/G-formulation excluded). Error bars denote standard error of the mean ($3 \leq n \leq 6$).

Figure 4. Linear regression plot of OCR as a function of matrix FBS concentration. The formulations studied consisted of 3.0% w/w sodium alginate solutions in pure 4.6% D-mannitol solution (G-0-G), pure foetal bovine serum (G-S-G) or a 1:1 mixture of those, all of which were combined with 2.5% w/w dispersions of calcium oligoguluronate particles with a calcium content of ~10%. Each data point is a mean OCR estimate as described in Fig. 2 ($3 \leq n \leq 4$). The regression slope was not statistically different from zero ($P = 0.26$).

containing Hank's balanced salts, cells immobilized in guluronate-rich gels depleted available oxygen stores in a shorter period of time, a significant effect of mannuronate content in the soluble alginate component ($P = 0.03$, $df = 24$, i.e. *all* formulations beginning with M tested against *all* formulations beginning with G). There was a tendency to higher OCRs in biostructures containing FBS (Figs. 3, 4). A single observation (Fig. 4, the OCR at ~60 fmol/h/cell for one of the 50% FBS parallels) was tested for being an outlier using Grubb's G-test. z -value calculated was 1.474, below the critical value of $z = 1.481$, and thus not significant at $P < 0.05$ level. It was therefore kept in the data set.

In the biostructure based on mannuronate-rich sodium alginate dissolved in Hanks' balanced salts, time taken to exhaust oxygen initially available was more variable than in the other formulations. This formulation also exhibited the only instances where this transpired more quickly in mannuronate-rich gels than in guluronate-rich gels.

Oxygen consumption rates

Based on oxygen concentration curves, continuous derivative oxygen concentration functions were determined for each run. Derivative at time points of interest was corrected for density of viable cells, and reported as the OCR. OCRs found were in the range of approximately 40–110 fmol/cell/h (see Figs. 3–5), most appearing to be in the region 40–80 fmol/cell/h (see Fig. 3). Interparallel differences were quite large, but cells in

Figure 5. OCR as a function of matrix alginate concentration. The formulations studied consisted of sodium alginate solutions in pure 4.6% D-mannitol solution or foetal bovine serum in concentrations of 1.5, 2.0, 3.0 and 4.0% w/w, which were combined with 2.5% w/w dispersions of calcium oligoguluronate particles with calcium content of ~10%. The formulations with 3.0% sodium alginate and a total concentration of 1.5% thus correspond to G-0-G and G-S-G (Table 1). Each data point is an OCR estimate as described in Fig. 2 ($3 \leq n \leq 5$). The slope of the fit to OCR data from cells immobilized in serum-deprived matrices was significantly different from 0 ($P = 0.027$), and also significantly different from the slope of the fit to OCR data from cells immobilized in serum-enriched matrices ($P = 0.014$).

most formulations displayed quite stable mean OCRs over the period in which data were recorded. Variability between adjacent points in the time series appeared to be less when the measurement interval was longer (data not shown).

Oxygen consumption rates were first investigated in biostructures consisting of sodium alginate cross-linked with medium chain weight calcium alginate particles (Fig. 3). In these formulations, it was found that oxygen consumption appeared lower in mannuronate-rich gels than in guluronate-rich ones, although differences were not statistically significant ($P = 0.93$), with respective mean OCRs at 62 ± 10 fmol/cell/h and 36 ± 6 fmol/cell/h. It was surmised that a difference might exist due to differential perfusivity of large soluble molecules, and to investigate this, a cross-linking agent had to be chosen to allow higher concentration of inorganic salts than that of the quite fast-gelling CaM particles. Calcium-oligo G particles have practical kinetics under physiological salt concentrations, and were chosen.

Medium chain weight CaM particles contribute significantly more to gel elasticity than shorter Ca-oligo G particles (data not shown). It was therefore judged to be necessary to increase overall alginate concentration to produce gels with comparable viscoelastic properties. The tendency of high-guluronate matrices to elicit

elevated OCRs compared to mannuronate-rich matrices appeared possible in the new formulations as well, with estimated mean OCRs for the two formulations being 90 ± 10 and 45 ± 6 fmol/cell/h respectively. Still, this was not statistically significant in the ANOVA with *post hoc* Tukey's analysis (Fig. 3).

Adding Hanks' balanced salts to the gel matrix appeared to have different effects depending on which alginate formulation was being used. In guluronate-rich biostructures, apparent reduction in OCRs was seen in biostructures containing Hanks' compared to those where gels' water phase consisted only of 4.6% D-mannitol as osmolyte (Fig. 3). Cells in guluronate-rich Hanks'-containing biostructures respired at 50 ± 10 fmol/cell/h. Comparison with respiration estimates in corresponding mannuronate-rich formulation (M-H-G) at 60 ± 30 fmol/cell/h is of little value due to high variability. This was not investigated further.

It appeared possible that oxygen consumption could be stimulated by addition of FBS to the gel matrix (Fig. 3, G-0-G versus G-S-G and M-0-G versus M-S-G). However, none of the differences are statistically significant. To further explore this trend, gels with intermediate concentration of FBS were formulated on the basis of 1:1 mixture of G-0-G and G-S-G, and linear regression analysis was performed with OCR as a function of overall fraction of FBS in the aqueous phase (Fig. 4). Slope of the regression curve was not significantly different from zero ($P = 0.26$, $df = 13$).

It was also investigated whether the alginate concentration would influence OCR (Fig. 5). Using linear regression with the concentration of sodium alginate as the independent variable, it was found that this tendency was statistically significant only in matrices *without* FBS ($P = 0.026$, $df = 14$). Additionally, it was tested whether slopes of linear regression equations for each condition (i.e. with and without FBS) were statistically different from *each other*, which was found to be the case ($P = 0.014$, $df = 25$).

It was hypothesized that gel elasticity and permeability to macromolecular substances might influence OCR, and elastic moduli of gels with various concentrations of soluble alginates were studied using an oscillatory rheometer. It was found that gels with added FBS achieved significantly higher elastic modulus, measured as a function of sodium alginate concentration ($P = 5.0 \times 10^{-3}$, $df = 5$) (G') in a significantly shorter time (Fig. 6). A plot of OCR as a function of gel elastic modulus is shown in Figure 7. It was found that the slope of a linear regression curve of OCR as a function of G' was statistically significant in gels without FBS ($P = 0.049$, $df = 2$) but not in gels with 50% FBS ($P = 0.32$, $df = 1$).

Figure 6. Kinetic studies of the elastic modulus (upper panels), viscous modulus (middle panels) and phase angle (lower panels) in matrices containing 0% (based on G-0-G, left panels) and 50% (based on G-S-G, right panels) added foetal bovine serum. The concentration percentage values refer to the alginate concentration in the syringe containing sodium alginate. The mean values of measurements of the elastic modulus as a function of time ($3 \leq n \leq 5$) were fitted to eqn (3), as detailed in the main text, and the values of A , the final elastic modulus are reported in the upper panels. In order to increase the legibility of the figures, every second data point in the time series has been omitted, and the scales on the abscissae are not the same in all panels. Matrices containing FBS achieved significantly higher values than matrices formulated on the basis of aqueous 4.6% D-mannitol ($P = 0.005$). Standard error of the mean is indicated by error bars when exceeding the size of the symbols.

Discussion

The OCR measurement system used in the present investigation was based on earlier work in our group (19), and a further development of the alginate gel system described there (unpublished data). As can be seen in Figure 2, there is an artificial elevation of the oxygen concentration at the earliest time points as sample prepa-

ration took place at room temperature and ambient atmospheric conditions, and sensor calibration is temperature-dependent. This effect was rapidly offset by equilibration with incubator conditions and respiration within the biostructures. Inclusion of Hanks' balanced salt solution in some of the formulations was intended as a control for presence of inorganic salts in FBS, but appeared

Figure 7. Oxygen consumption rates as a function of final matrix elasticity in matrices without (based on G-0-G, open circles) and with (based on G-S-G, filled squares) added FBS, as determined by the A parameter in equation. OCR appears to fall as elastic modulus increases. It should be noted that the rheological data do not reflect the actual properties of the gels while OCR data were being recorded, as the rheological parameters shown here were calculated on the basis of measurements on gels which had not been incubated with growth medium, whereas this was a necessary treatment in the oxygen measurement system. It should be expected that access to more salt would increase the final elastic modulus, particularly for gels not containing FBS. The slope of the linear regression curve for matrices without FBS was significantly different from zero ($P = 0.037$), but the two regression slopes were not statistically different from each other ($P = 0.5$). Each data point is based on the mean of at least three different experiments for both parameters ($3 \leq m, n \leq 6$). Error bars denote standard error of the mean.

to have partly opposite effects to those of including serum. At least, it seems clear that inclusion of inorganic salts did not in itself increase OCR.

All time series showed a nearly linear reduction in oxygen concentration after temperature stabilization (data shown for two formulations in Fig. 2), which is consistent with the postulation that oxygen from diffusion would not significantly impact calculated OCR (19). However, as noted, for some oxygen concentration curves, OCRs seemed to vary over the course of the experiment, resulting in deviations from a linear model. What might appear as reduced OCRs may be a result of increased influx of oxygen due to diffusion, as concentration gradient increased. On the other hand, apparent increases also took place on a time scale that could allow for expansion in number of cells, a possibility that was regarded as outside the scope of the current study.

In the time frame chosen for calculation of OCRs, i.e. ~2–5 h after immobilization, there seemed to be differences between several of the formulations, as

postulated (Figs. 3–5): Although not completely conclusive, our data altogether seem to support higher respiration rate of entrapped T-47D cells in the presence of higher serum concentrations and in more open gel networks. A more diffusion permissive, open alginate network will be present in guluronate-rich gels or if the alginate concentration is reduced (17,23). The effect of molecular permeability through the gel network on respiration rates may be supported by higher OCR values when guluronate-rich alginate is used for otherwise comparable samples (Fig. 3: G-0-M versus M-0-M, G-0-G versus M-0-G, G-S-G versus M-S-G) and when alginate concentration was reduced (Fig. 5).

The alginate gel network created using Ca oligo-G blocks as gelling ion source could also be expected to have lower alginate density due to lower concentration of polymer chains containing multiple polyguluronate segments. According to the previous reasoning, this would indicate lower OCR (G-0-G versus G-0-M and M-0-G versus M-0-M). A lower OCR floor for cells, less responsive to such factors, may perhaps relate to the lowest OCR measured here at about 40 fmol/cell/h.

The relatively low number of samples and experimental variations may, however, explain lack of significant difference between several of the conditions studied, making the current data more indicative. Also, there were no indications of alginate gel network-dependent effect where Hanks' balanced salts was used as solvent for sodium alginate component of the gel system (G-H-G versus M-H-G and M/G-H-G) and perhaps other mechanisms related to this solution may play a role. The viabilities of cells in the different formulations were not statistically different, but OCRs were nonetheless calculated using the per-formulation viabilities, as it has been shown that when cell suspensions are ejected through syringes, viscoelastic properties of the suspension medium influences viability (24).

T-47D cells used in the present experiments are routinely cultured with 10% FBS present in the culture medium. It has been demonstrated that these cells are growth-inhibited in the absence of serum (25), and in this and previous studies, we observed significantly lower respiration rates than those found in monolayer cultures in most protocols (19,21). However, the OCR found in guluronate-rich gels with high serum content (Fig. 3) is closer to the monolayer range of 177–227 fmol/cell/h previously found, with approximately 100 fmol/cell/h for two of the formulations studied here (G-S-G and G-0-G), versus only about 60 fmol/cell/h in our earlier report (included here as G-0-M). The possible effect of serum factors increasing OCR of cells in higher porosity gel networks is supported by the change in

trend seen in Figure 5 and potentially by the mostly higher OCR values (although not statistically different) seen between comparable samples in Figure 3 (G-S-G versus G-O-G and G-H-G) and Figure 4. Change in gel structure and molecular permeability will, however, also change gel structure elasticity which may perhaps also influence cell respiration rate (Fig. 5). Wang *et al.* (26) may have observed a similar phenomenon in the case of rat osteoblasts, although there appear to be some calculation errors in their report, making this difficult to verify. A well-conducted study of OCR in neural stem cells cultured in suspension as aggregates and in microcapsules showed similar disparities (27), and a study of cells undergoing change in environment from native 3D to 2D expansion showed a strong, opposite effect (28).

In the present system, counting gelling components, including the dead volume of syringes and cell suspension, the total volume of the biostructure mixture was 1300 μl . Of this, 100 μl was cell suspension, based on growth medium supplemented with 10% FBS. Thus, even formulations where no extra FBS had been added contained approximately 0.8% FBS. Further serum components could also be available through diffusion from the layer of growth medium residing on top of the biostructures. The present biostructures are rather large compared to physical dimensions of devices commonly used in 3D culture – at least when compared to microcapsular devices – and have lower cell density. This might increase effects of diffusion-limited supply of large soluble factors, as the mean diffusion path is increased. As demonstrated earlier, oxygen concentration throughout the biostructure remained uniform for biostructures cultured under conditions similar to those in the present work (19), while it is well known that ~2% w/w alginate hydrogels do not significantly influence diffusion of oxygen compared to pure water (29). Medium chain weight CaM calcium alginate has alginate chains of sufficient length to substantially add to the gel network. It may thus result in a more tortuous gel network when present to the amount of 1.0% w/w than a greater weight fraction (1.25% w/w) of low chain weight Ca oligo-G calcium alginate particles.

Further investigations, as shown in Figure 5, indicated that OCR decreased when alginate concentration was increased, if only very little FBS was present. It was not possible to determine whether OCR increased when increasing concentrations of FBS were added to the matrix (Fig. 4). Taken together, this might imply that amounts of FBS components available to cells influenced OCR, especially as growth factors induce proliferation in T-47D cells (25), and proliferation drives OCR (21). Limiting effect on availability of growth factors as a result gel network-dependent

diffusion may, to an extent, have been overcome by increasing concentration of FBS in the matrix. However, further studies quantifying molecular permeability and identifying specific metabolic drivers, will be needed to confirm this hypothesis. Our choice of a linear model only serves to make the point that a difference between different matrices exists, that depends on alginate concentration. Further studies will also be needed to determine whether other models describe the correlation better.

To the best of the authors' knowledge, little is known about how viscoelastic properties of the surrounding matrix may give direct respiratory cues to immobilized cells. Studies have shown that when anchorage motifs are present on the hydrocolloid backbone, matrix elasticity influences differentiation and proliferation of cells in 3D culture (30), and also may play a role in tumour malignancy (31). Rheological properties (Fig. 6) of the matrices for which respiration data were shown in Figure 5, demonstrated significant differences with respect to gelation kinetics and final gel elastic modulus. In Figure 7, OCRs from Figure 5 are plotted as a function of final matrix elasticity. This indicates an interesting tendency, which in our opinion warrants further investigation. Also, as above, correlation need not be best described by a linear model: For matrices containing little FBS, there was no trend in OCR values within elasticity interval of approximately 200–800 Pa. However, it must be noted that geometries used in respiration measurements and the rheological measurements were not identical.

It has been shown that the source of sera used in cell cultures is unlikely to influence oxygen metabolism (32). This is useful background information as human serum is seldom used in research of this type. As noted, alginates of varying sequence composition may have differential diffusive properties: Alginates high in guluronic acid are generally more permissive towards diffusion of macromolecular substances. Guluronate-rich gels, whether in the form of capsules or macroscopic gels, are more permissive to diffusion (17,23,33). Attempts have also been made to generalize diffusional properties of alginate gels based on monomeric composition and concentration (23).

Comparing OCR in guluronate-rich versus mannuronate-rich alginate matrices showed significant differences. Previous work has also shown that composition of alginates used for cell immobilization has an influence on oxygen consumption, although our experiments seem to contradict these results: Stabler *et al.* (16) found that mannuronate-rich alginates elicited higher rates of metabolism in microencapsulated βTC3 murine insulinoma cells, in comparison to guluronate-rich alginates,

when studied over significantly longer periods of time, than in the present study. Mannuronate-rich beads tend to shrink to a greater extent than guluronate-rich beads (34). This could possibly result in concentration-dependent effects on metabolism, as local cell density would increase. This does not seem a likely explanation in the present case, as no significant gel shrinkage was observed.

On the other hand, Mukundan *et al.* (35) have shown similar disparities in the case of glucose and oxygen consumption in microencapsulated β TC3 cells compared to monolayer cultures. In their experiments, OCR of encapsulated cells was permanently reduced by about 30%. In spite of this qualitative similarity, capsule geometry, suspension of capsules in growth medium and cell line used in their experiments make further comparison difficult. It is interesting to take into account that the same group later showed that encapsulated β TC3 cells were able to proliferate extensively (36).

Cell–matrix interactions have been reported by Guaccio *et al.* (37) to significantly affect OCRs of chondrocytes immobilized in agarose compared with collagen gels. The effect observed in their experiments was quite similar to that observed in the present work, as they registered OCR of 211 fmol/cell/h in agarose and 94 fmol/cell/h in collagen gels, but the cause was altogether different: higher OCRs were probably a result of lacking anchorage motifs in the matrix and could be *diminished* by addition of soluble RGD peptides, whereas none of the presently investigated gels contained anchorage motifs. Nonetheless, this provides another instance of clear differences in OCR as a direct function of matrix composition. Culture history and exogenous differentiation pathways have been shown to significantly influence OCR of cells in monolayer and pellet culture (28,38). Some authors have previously pointed to possible influence of gradients of growth factors or nutrients within bioconstructs on entrapped cells' metabolism, phenotype and function (39,40).

To the best of our knowledge, no similar results displaying effects of presence of growth serum on OCRs of cells cultured in a 3D environment have been published. The present results supplement previous studies of microenvironmental respiratory modulation in highlighting the importance of chemically well-characterized matrices when devices in regenerative medicine and biostructures for other purposes are designed. Although dimensions of the present biostructures are atypical compared to those common in 3D culture applications, it is possible that crucial growth factors or other macromolecular factors should be identified and included in the assembly of cell immobilization matrices.

In conclusion, results presented above may indicate that observed differences between monolayer and 3D cultures in respiration of T-47D cells, is at least partly due to diffusion-limited supply of growth factors present in growth sera frequently used in cell cultures. Addition of FBS at high concentration led to increase in OCRs of T-47D cells immobilized in guluronate-rich alginate hydrogels, as did reduction in elastic modulus. This may have important implications for design of 3D culture devices, especially those which do not incorporate some method of perfusion.

Conflict of Interest

None.

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