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Review

Application of microdialysis in tissue engineering monitoring

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Abstract

In this review article, the microdialysis for tissue engineering monitoring is discussed. Among various monitoring techniques, microdialysis is advantageous for its capacity of perfusion on-line, and off-line multiple parameter monitoring. Following a description on the general system and performance, the main challenges to apply this technique for reliable long term monitoring are outlined. Further opportunities are identified.

Keywords: Microdialysis; Tissue culture monitoring; Tissue engineering

1. Tissue engineering

Tissue engineering (TE) is to develop biological substitutes for implantation into body and/or to foster tissue regeneration and remodeling, with the purpose being to replace, repair, maintain, or enhance function. The development of engineered tissues usually involves the seeding of selected cell type into gels (e.g. collagen, agarose, alginate and fibrin) [1] or a three dimensional (3-D) biodegradable scaffold and culturing such a construct under desired conditions in a tissue culture bioreactor [2]. In the bioreactor, cells proliferate and differentiate to the correct phenotype and secret extracellular matrix (ECM) macromolecules, which assemble and accumulate to form the tissue. The cell ECM gradually replaces the scaffold materials, while the starting scaffold is degraded, resorbed, or metabolised [3–5].

From the first clinical practice of tissue engineered skin [6,7] to engineered blood vessels [4] and even engineered bone [8], cartilage [9], heart components [10], liver [11] and most other tissues, TE research has achieved remarkable progress. However, at present TE is still in experimental stages for virtually all tissues and organs with vigorous investigations into the role of factors such as cell source [8,12], scaffold type and organisation [13–15], growth factor addition [16] and mechanical stress in promoting tissue growth [17,18].

2. Monitoring in tissue engineering

The process of growing cells in 3-D structures to form engineered tissue and organs using tissue engineering methods is long, taking weeks or even months to complete [19–21]. Thus, it is vital to monitor the cell and tissue status throughout the whole tissue during the culture period, preferably on-line with non-destructive methods. Current methods for monitoring tissue culture, apart from measuring the overall and averaged indicators in the effluent of the bioreactor, are largely based on destructive biochemical or histological methods, although non-destructive methods such as MRI or micro-CT have been attempted. For instance, normal methods to assess cell growth and tissue formation are based on the destructive histology. The constructs are taken out of the bioreactor and sliced for various biological and biochemical assays such as cell number, viability and/or extracellular matrix components like aggrecan or collagens using immunohistochemical assessment or biochemical assays [22,23].

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This is acceptable to assess the quality of the final products for quality control, but it would be far from ideal to monitor the cell growth and tissue formation during the tissue culture. The monitoring of tissue development is usually done by analysing the metabolic products in the bioreactor effluent, but this can only indicate the collective cell activity within the whole construct and will not be able to study the cell response to local environment changes or differentiate due to, for example, mass transfer limitation to nutrient and oxygen supply [24].

The nutrient supply and waste removal to and from the cells inside the construct rely on diffusion in most engineered tissue cultures. Mass transfer limitation is hence a major problem if bulky tissue is to be produced as desirable for some clinical applications. Cell inside will not proliferate, differentiate or even die as occurs in tumour spheroids [25]. This becomes more severe as the tissue culture progresses, as cell density and extracellular matrix density near the construct surface increase. In many cases, the cultured tissue would be highly heterogeneous with a high cell density near the surface and very low or even none cellular activity in the central area [24]. This is a common problem for any bulky tissues without vascular networks. Therefore, non-destructive methods which could monitor changes in cell metabolism or viability or even tissue deposition locally within the construct and could point out adverse responses early during culture would prove invaluable. At present, non-destructive monitoring throughout the construct can be achieved using technologies such as NMR [26,27], MRI [28,29] or optical methods [30] to *in vivo* study of metabolism. These techniques can provide useful but limited information on spatial variations in tissue status, but cannot achieve long term and continuous monitoring of cellular activity and tissue functionality. Moreover, their use is limited by cost and availability.

Here, we propose an alternative method of on-line monitoring of tissue engineered constructs using the principle of microdialysis to determine local production of metabolites and tissue components in the extracellular fluid of the construct. It is a minimally invasive and non-destructive method, which should not interfere with the normal development of the tissue formation.

3. Microdialysis: on-line monitoring technique

Microdialysis is performed by perfusing a small semi-permeable hollow-fibre membrane probe inserted into the tissue with a physiological fluid (the perfusate). Molecules outside the membrane in the extracellular fluids will diffuse through the membrane due to the concentration gradient if they can pass the pores. The solution that exits the probe, the dialysate, which contains the marker molecules, can be collected for analysis. The size of the molecules diffusing through the semi-permeable probe membrane depends on pore size (Fig. 1). The first microdialysis experiments were conducted as a method to measure dynamic release of substances in the brain and blood plasma [31]. It has since been used to study

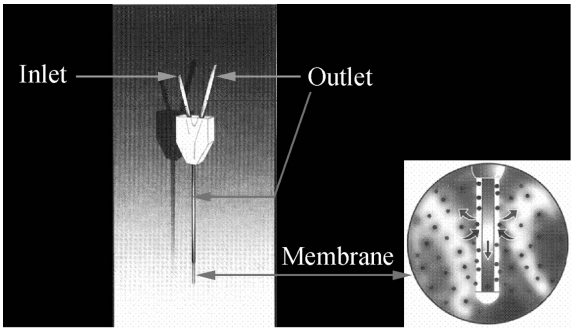

Fig. 1. Illustration of a microdialysis probe.

metabolism in numerous tissues of living animals and human subjects such as brain [32], muscles [33], subcutaneous adipose tissue [34], lungs [35], kidneys [36] and liver [37]. Microdialysis is widely used for pharmacokinetic research [38] and has also been used to monitor cell metabolites in cell culture medium [39]. The most important feature of microdialysis is that it allows sampling of the extracellular fluids. It has been reported that there is no major tissue trauma inducted by the microdialysis catheter.

Most tests with microdialysis typically use membranes of a molecular weight cut-off (MWCO) around 20 kDa to exclude bigger molecules and hence to simplify the analytical procedures [32,33,40]. Nevertheless, many bio-molecules such as cell mediators, cytokines or growth factors are bigger than 20 kDa. Obviously, microdialysis probes with lower MWCO are not suitable for the collection of big bio-molecules. However, recently membranes with a higher MWCO which allows the monitoring of a wide range of soluble molecules have been used for short-term sampling of peptides and macromolecules in muscle, adipose tissue and dermis [41–46].

3.1. Probe characteristics

Multiple sample parameters will dictate the choice of microdialysis probes as well as membranes that are used for *in vitro* and/or *in vivo* sampling. Based on the principle of microdialysis, the size of the molecules diffusing through the semi-permeable probe membrane depends on pore size. Molecules smaller than the membrane pores can diffuse into inner fibre lumen and be carried to the outlet by the continuously flowing perfusion fluid. Larger molecules will either be completely rejected by the membrane pores or diffuse so slowly through the membrane causing their recovery to be negligible. Therefore, membrane pore size is the most critical factor for probe selection for non-charged membranes. Theoretically, the MWCO of microdialysis membrane three times greater than the molecular weight of the target molecule is sufficient to permit diffusion across the dialysis membrane [47]. The recovery of membrane (definition see Section 3.2) is directly proportional to membrane length and diameter, the greater the membrane area the higher the recovery. Ideally it would be desirable to use

long membranes to maximize recovery, but unfortunately this is not always possible as the tissue being studied may be anatomically limiting. Typical lengths and width of probes commercially available are 1–30 mm and 200–500 μl outer diameter (o.d.), respectively. Membrane material is also an important part of the sampling device in a microdialysis experiment. Analysts must select a membrane that not only achieves a high extraction fraction (definition see Section 3.2) but also is compatible with complex matrix conditions, pH, and temperature [48]. A variety of hollow-fibre membranes with different surface morphologies, molecular weight cut-offs, and materials of construction are commercially available, such as polysulfone, polyamide, polycarbonate–polyether copolymer, cuprophan and polyethersulfone. Perfusion rate is another parameter to affect recovery both *in vitro* and *in vivo*. As would be expected the probe recovery varies inversely with perfusion rate [49]. For example, a slow perfusion rate of 0.2 μl/min allows a longer time for the perfusate to equilibrate with the solution surrounding the probe, achieving close to 100% recovery. However, low perfusion flow rates may be hampered by problems associated with sample evaporation as well as poor temporal resolution [50]. Therefore, generally flow rates are applied between 0.2 and 5 μl/min for most applications of microdialysis [46].

3.2. Membrane recovery

The dialysing properties of microdialysis membrane are routinely expressed as its recovery for a particular solute. The recovery of the probe can be evaluated by comparing the concentration of the solute of interest in dialysate and in probe surrounding, expressed in terms of relative recovery (RR). This is represented as

$$\mathrm{RR} = \mathrm{Cd}/\mathrm{Ce} * 100\%, \quad (1)$$

where Ce is the concentration of a given substance in probe surrounding, e.g. in extracellular fluid, and Cd is that of same solute in the dialysate.

The concentration of solute in dialysate can be equal to the solute concentration in probe surrounding if flow rate of perfusate equals 0. Otherwise, the concentration of the solute in dialysate will always be lower than that in probe surrounding.

Another parameter described for the performance of the microdialysis membrane with respect to analyte mass transport is the extraction fraction (EF), as shown in

$$\mathrm{EF} = 1 - \exp[-1/Q_\mathrm{d}(R_\mathrm{d} + R_\mathrm{m} + R_\mathrm{ext})] \quad (2)$$

where Q_d is the perfusion flow rate; and R_d, R_m and R_ext are the resistances to the dialysate, membrane and external factors (such as tissue, solution or particles in a complex matrix), respectively [51,52].

EF is more commonly known as RR, as defined in

$$\mathrm{RR} = 100\mathrm{EF}. \quad (3)$$

Microdialysis EF is a complex function of the solute mass transport properties through the sample, the membrane, and the perfusion fluid. *In vitro* diffusion of a molecule into the probe is given by the diffusion coefficients in solution and across the probe membrane. Because R_m is much greater than R_d and R_ext for *in vitro* sampling process, the permeability factor generally is reflected by the resistance of the membrane to the transport of analytes ($R_\mathrm{m} \gg R_\mathrm{d} \gg R_\mathrm{ext}$) (Fig. 2a). However, *in vivo* diffusion to the probe through the tissue is hindered, as substances must undergo a tortuous diffusion path around cells to reach the membrane. Thus, *in vivo* the resistance through the tissue is dominant ($R_\mathrm{ext} \gg R_\mathrm{m} \gg R_\mathrm{d}$) and the drop in concentration between surrounding and dialysate occurs across the tissue (Fig. 2b). This was demonstrated experimentally where three different membrane types were found to have similar *in vivo* recoveries despite different recoveries *in vitro* [51].

3.3. Probe calibration

3.3.1. In vitro recovery

The simplest microdialysis calibration method is to determine *in vitro* the dialysing properties of the microdialysis probe for the particular substance(s) of interest. Ungerstedt et al. first recognized that the dialysate concentration was governed by probe recovery [53]. *In vitro* recoveries are routinely used to calculate extracellular concentrations, based on the assumption that recovery for a given probe is constant regardless of the sampling environment [54]. However, increasing evidence has proved that it is less reliable to introduce such a value to *in vivo* studies [55]. *In vivo* recoveries are expected to be lower than *in vitro* as tissue properties surrounding the probe reduce the mass transport of substances to the membrane. Obviously the biological factors, such as the tissue tortuosity,

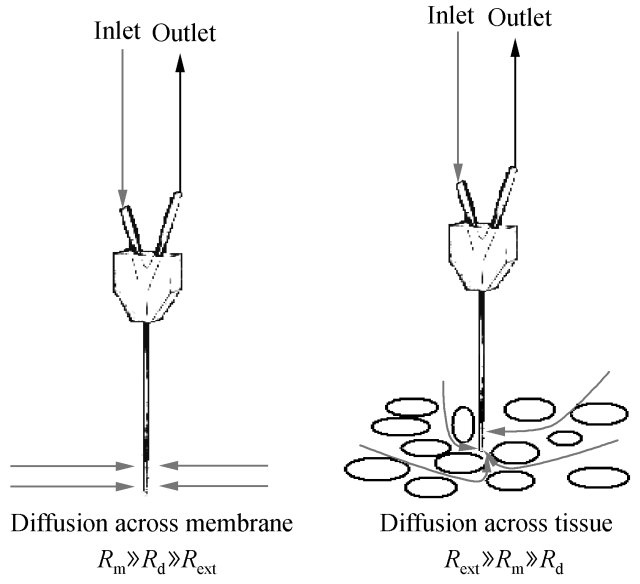

Fig. 2. Schematic representation of solute diffusion *in vitro* and *in vivo*.

free fluid space between the cells, metabolism, uptake and release, as well as intracellular and extracellular exchange should be taken into account for *in vivo* calibration.

3.3.2. Zero-net-flux (ZNF) recovery

The most favoured empirical method of determining *in vivo* recovery and extracellular concentrations was the zero-net-flux recovery, and was first described by Lönnroth for human subcutaneous measurement of glucose [56] and adenosine [57]. In this method the solute to be measured is added in known concentrations to the perfusate. The direction of diffusion flux of the solute is then either into or out of the probe, depending on where the higher concentration is present. Perfusate concentrations are chosen to balance the expected extracellular concentrations, so there will be a point at which there is no net flux across the membrane where the probe neither loses to nor gains from the probe surrounding. At the equilibrium point of ZNF, the concentration present in the perfusate is equal to the *in vivo* extracellular concentration. The extracellular concentration and *in vivo* recovery are determined by plotting the difference between perfusate and dialysate concentrations against perfusate concentrations, the zero point on the *y*-axis yields the extracellular concentration and the gradient of the line is equal to the *in vivo* probe recovery.

Values for the extracellular concentration and *in vivo* recovery by ZNF method are obtained by direct measurements and no assumptions about the tissue sampled are made. However, it has been only applied in practice for the determination of extracellular concentrations of metabolites for a given solute, such as glucose [58,59], lactate [60], ascorbate [61], dopamine [62] and glutamate [63]. There are no wide applications of this method to large molecules, and also it takes time to do a series of experiments to get the *in vivo* recovery, and it cannot be applied to measure unknown or uncertain molecules.

3.3.3. Internal reference recovery

The internal reference calibration was done essentially as described by Scheller and Kolb [64] and then has been widely used *in vivo* probe calibration [65–70]. Since diffusion across the membrane is bi-directional it is possible to add to the perfusate a known amount of an identifiable marker that is chemically and biologically same or similar to the solute of interest, and monitor its loss when perfused through the probe. The relative loss (RL) is a measurement of a standard solute diffusing from the probe perfusate into the surrounding interstitial fluid. It is

$$\mathrm{RL} = (C_\mathrm{p} - C_\mathrm{d})/C_\mathrm{p} * 100\%, \quad (4)$$

where C_p is the concentration of a reference in the perfusate (e.g. fluorescent-labelled or radio-labelled substances), and C_d is the concentration of the same solute in collected dialysate.

RL is assumed to be equal to relative recovery (RR) if the solute taken as internal standard has the same or similar molecular size and diffusion properties as the solute of interest, seeing Eq. (1). Knowing RR, C_e can be readily determined from the measured C_d.

3.4. Microdialysis sampling for proteins

Detection of macromolecules is highly challenging using micro-membrane sampling because their large size and thus low diffusion coefficients cause mass transport limitation through the probe resulting in a lower recovery. In addition to this, non-specific adsorption of protein molecules to dialysis membrane (fouling) and the low *in vivo* concentration of macromolecules lead to the reduction of sensitivity on protein sampling and analysis. Overcoming membrane fouling and improving recovery are essential requirements for macromolecular marker sampling with large MWCO membranes.

From Eq. (2) we can see that decreasing flow rate and/or increasing membrane area contribute to the enhancement of substance recovery. However, most obvious parameters such as the length and the width of probe are fixed at a range of 4–10 mm and 200–500 μm, respectively, to achieve minimal invasion particularly for the application of microdialysis *in vivo*. The perfusion flow rates are typically 0.3–2 μl/min when employing microdialysis with high MWCO membrane. PES is commonly chosen as the material of membrane especially for sampling macromolecules owing to its biocompatibility [71]. Therefore, attempts and effect on changing of membrane parameters to enhance recovery for microdialysis approaches *in vivo* and/or *in vitro* are limited.

Nevertheless there are still some approaches described for solving these problems. To overcome the problem regarding poor sample volume retrieval and protein recovery, two methods have been described so far to preserve sample volumes: (i) the use of a high molecule weight substance e.g. dextran-70 [72,73] or bovine serum albumin (BSA) [74] in the perfusate to produce osmotic balance, and (ii) using ultrafiltration in microdialysis procedure either by push–pull pumping system [44,75] or hydrostatic pressure by lowering place of the probe tubing outlet [45], which however does create net fluid removal through the membrane. This prevents excessive loss of perfusate through the membrane pores out into the surrounding, and increases protein yield. Another attempt to enhance the protein recovery is to use affinity beads in the perfusate which proved to dramatically increase the recovery of target proteins [76]. Decreasing flow rate to a certain extent may achieve higher recovery [77].

3.5. Assessment of probe membrane fouling

Fouling has always been a problem when using membranes in complex systems. Torto and co-workers showed that most membranes used for microdialysis sampling exhibit some degree of interaction with proteins [47]. As mentioned in Section 3.2, the molecules that diffuse through saline into a probe *in vitro* are completely different from the molecules that diffuse through tissue *in vivo*; hence

the degree of fouling that occurs *in vitro* and *in vivo* may differ. *In vitro* fouling may cause from saline concentration polarisation if flow rate is relatively high, however, fouling *in vivo* is likely to occur through adsorption of species on to the surface and within the pores of the membrane. There are few studies on probe fouling during *in vivo* applications of microdialysis. Boubriak et al. [78] have reported *in situ* monitoring membrane fouling by monitoring changes of phenol red (PhR) concentration in dialysate during engineered tissue culture. PhR could be a good candidate for the monitoring of possible changes in probe recovery because it is routinely present in culture media at constant concentration as a pH indicator and is not consumed by the cells during the experiment. Therefore, the RR of phenol red measured by the probe can be used for assessing the occurrence and the extent of membrane fouling.

4. Microdialysis for tissue engineering monitoring – case studies

Microdialysis probes have been used to monitor 3-D tissue growth [78,79] and tissue explants culture [80], by picking up soluble molecular markers which indicate the cell viability, type, functions and extracellular matrix turnover. The possibility of monitoring chemical gradients within a tissue engineered construct is also attempted to determine local changes in cell metabolism [78,79]. Methods of calibrating the probes *in situ* and identifying any possible probe fouling have been developed using phenol red (present in the culture media), radioactively labelled methyl-glucose, and fluorescently labelled dextrans [78–80].

4.1. Experimental method

In the engineered cartilage case, a cylindrical construct consisting of bovine articular chondrocytes in a 3-D matrix was formed in a bioreactor. One or more micromembrane probes were inserted into the construct at known locations relative to the nutrient supply. For the explant tissue study, intact bovine intervertebral discs dissected from adjacent vertebral bodies were similarly inserted into the bioreactor. The top and bottom surfaces of the construct or disc were perfused with tissue culture medium to supply the cells with nutrients and to remove metabolic wastes. Nutrient and metabolite gradients formed within the 3-D construct; concentrations of nutrients and metabolites depending on the balance between rates of cellular activity and rates of nutrient/metabolite supply or removal. Nutrient and metabolite concentrations at defined locations within the construct were continuously monitored using the microdialysis probes. Responses to culture perturbations known to affect chondrocyte metabolism such as changes in medium pH or osmolarity or serum concentration were monitored. Fluorescent dextran and phenol red were used to determine *in situ* the relative recovery of the solute of interest, and to detect membrane fouling, respectively. The remaining dialysate was pooled daily and the samples were analysed and quantified by FPLC and identified using SDS–PAGE and Western blotting. At the end of the culture period (up to 14 days), the construct or disc was snap frozen and assayed for spatial variations in cell viability, cell number and GAG and collagen concentrations.

For measurement of concentrations of low molecular weight solutes (<1 kDa) in alginate gels or alginate–chondrocyte constructs, autoclavable microdialysis probes of standard design [78,79] formed from polyethersulfone (PES) dialysis membrane with 15 kDa cut-off and with an effective length 4 mm, outer diameter 0.6 mm and with a 35 mm flexible polyurethane shaft were used. For monitoring macromolecular markers [80], CMA/20 probes with 100 kDa MWCO membrane and with an effective length 10 mm, outer diameter 0.4 mm and with a 25 mm flexible polyurethane shaft were used.

Probe recovery in our experiments was expressed as a percentage of the concentration of the solute in the probe (the dialysate) relative to that in the surrounding external solution (relative recovery). In the experiments, *in situ* probe relative recovery was assessed on the basis of the percentage of phenol red in the dialysate after equilibration of the construct with DMEM containing phenol red [78–80]. In this way the relative recovery of the probe was continuously monitored during the experiment to identify any membrane fouling. As the relative recovery depends on molecular weight of the molecules, several tracer molecules were used to determine the relative recovery of molecules of similar size, including Florescent dextran (40 kDa, for protein markers) [80] and 3-methyl-glucose (for glucose and lactate) [79].

4.2. Measurement of concentration-gradient, monitoring cell metabolic activities using 15 kDa microdialysis probe

To determine whether concentration gradients of important metabolites could be accurately assayed, eight probes, positioned in a helix with a distance of 1.64 ± 0.17 mm between them, were introduced into a cell-free construct of 14.8 mm height maintained at 37 °C. At a known time, 10 mM lactate or 0.06 μCi/ml 3-methyl-glucose was introduced into the buffer perfusing the top surface while flow along the bottom of the gel was stopped. The dialysate was collected over 27.5 h and the concentration of the solutes in dialysate was determined by an appropriate assay. Results are shown in Figs. 3 and 4 [79]. The changes in lactate concentration from probe to probe could be distinctly determined giving an apparent diffusivity ratio through the gel + probe of 0.42 ± 0.03 relative to diffusivity in aqueous media. The time-course of change in lactate or glucose concentration at different positions in the gel followed that calculated from diffusion theory (Fig. 4), validating the use of these probes for determining concentration gradients through the gel. The effect of glucose concentration, osmolarity (Fig. 5) and addition of fetal bovine serum (growth factors) (Fig. 6) on the cell metabolic activities and their

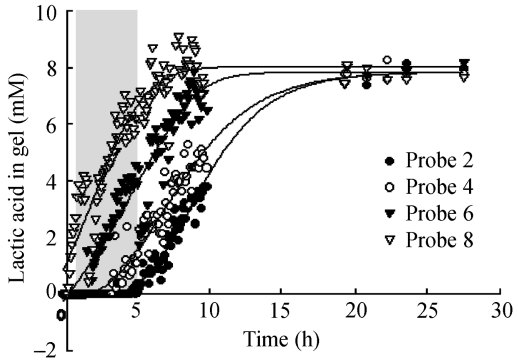

Fig. 3. The time-course of lactate concentrations in the gel measured at four probes (for clarity), with probe 2 closest to the perfusing surface and probe 8 farthest from it. Results in shadow were used for estimation of solute diffusivity.

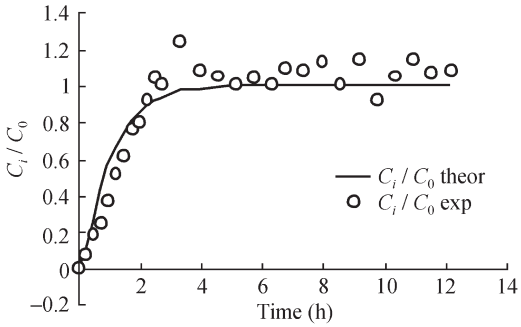

Fig. 4. Calculated versus measured time-course of lactate concentration in the construct centre.

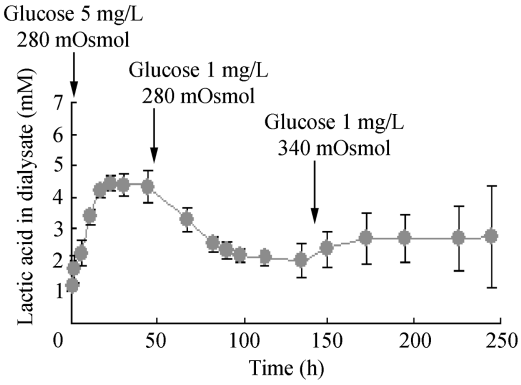

Fig. 5. Glucose and osmolarity affect chondrocyte metabolic activity as measured by micro-membrane probes.

distribution as well as their transient was successfully studied [78]. Limitation of metabolic activity by nutrient depletion was clearly identified in the thicker constructs. Low glucose concentrations or high levels of lactic acid in the construct centre were found to be linked with loss of cell viability and low rate of matrix accumulation [78]. In large constructs (>4 mm) viable cells were mainly concentrated near the nutrient supply unless the construct was seeded at very low cell densities ($<2 \times 10^6$ cells/ml). Glycosaminoglycans were located around the areas containing live cells [79].

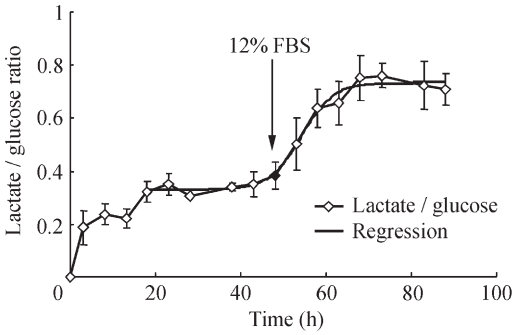

Fig. 6. Simultaneous monitoring of glucose and lactate and the effect of growth factors (FBS).

4.3. Sampling chitinase-3-like protein 1 (CHI3L1) using 100 kDa microdialysis probe

Sampling macromolecular markers are necessary for the monitoring of extracellular turnover and other specific functions such as protein synthesis. To investigate whether small metabolites ($M_W < 1$ kDa) and soluble macromolecules ($M_W > 20$ kDa) can be monitored for a longer period of time using a single probe, whether the measurements were stable and reliable over time and whether the probe was fouled by external proteins, we used a commercial microdialysis probe of 100 kDa MWCO membrane and a bovine intervertebral disc (IVD) explant as the model tissue for the test. We cultured the IVD explant for 7 days and measured the levels of metabolites and characterised soluble proteins in the dialysate using FPLC. Fluorescent 40 kDa dextran and phenol red were used to determine the relative recovery of the solute of interest, and to detect membrane fouling, respectively. We identified three soluble matrix proteins, which we found were clearly related to the cellular activities of chondrocytes and were responsive to the changes in culture condition. They were more indicative of chondrocyte metabolic activity than the pro-collagen peptide which is regarded as a classical marker. Monitoring of phenol red content in the dialysate indicated that there was no significant fouling of the membrane probe during a 7-day culture period and the relative recovery of macro-

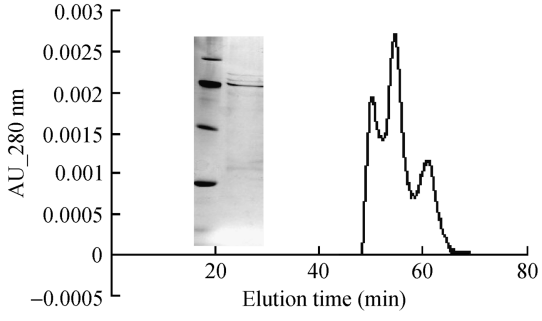

Fig. 7. Daily changes in the concentration of three proteins in the dialysate during 1-week IVD culture. The proteins were assayed by FPLC.

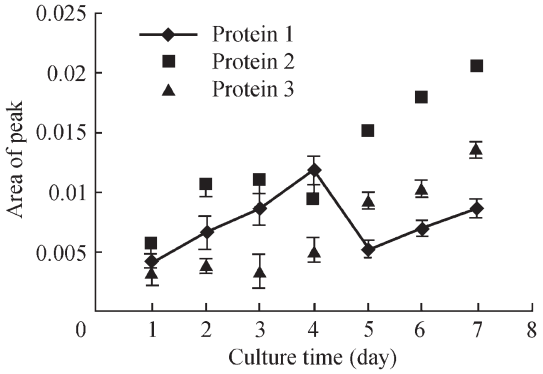

Fig. 8. Protein assays using FPLC and SDS–PAGE with silver stain (left inset). Lane 1: molecular mass standard (Bio-Rad); lane 2: crude dialysate.

molecules of interests remained roughly stable at 9%. Figs. 7 and 8 show the analyses of dialysates from a 100 kDa micromembrane probe when used to monitor cellular activities in IVD culture [80]. One of the three proteins has now been identified successfully as chitinase-3-like protein 1 (CHI3L1) using mass spectrometry and Western blotting (antibodies kindly supplied by Dr. A. Recklies, Shriners Inst, Montreal). It appears to be a sensitive marker for chondrocyte and cartilage metabolism. The detailed biological link of these protein markers to cell/tissue functions, and the reason a sudden drop in protein 1 expression on day 4 merit a further detailed study.

5. Future opportunities

Microdialysis can be successfully adapted as a reliable technique for the monitoring of cell metabolism and tissue functions. Protein synthesis and energy metabolism within the functional tissue can be continuously sampled using microdialysis probes of high molecular weight cut-off. The membranes do not get fouled significantly, and *in situ* methods to identify fouling have been developed. The system has thus been successfully used to monitor metabolism within cultured tissue explants and could be applied to monitor tissue growth in engineered tissues or during tissue repair *in vivo*. Further research on the engineering side includes predicting the relative recovery and hence eliminating the need for *in situ* calibration. Another exciting area is to develop a new type of membrane probe, which can be integrated into the tissue engineering scaffold.

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