Introduction
Measuring analyte concentrations, such as calcium and nitric oxide, in the intracellular environment has become an important research area, enhanced by the application and development of fluorescent probes and fluorescent analogues [1] and the increasingly economical availability of optical imaging instruments such as confocal microscopes. Alongside these developments, biotechnology and nanotechnology have been making a great impact in the area of sensors with micron and submicron dimensions suitable for intracellular measurements [2]. The nanosensors known as PEBBLE (photonic explorers for bioanalysis with biologically localized embedding) derive their origins from the pulled fibre technology developed by Tan et al. [3], in turn a development from the optode.

However, optical fibre optodes and fluorescent probes display a number of disadvantages for intracellular measurement. Optodes are invasive to the cellular system, the technique requires highly skilled personnel to position the optode in the cell and the resultant signals are not spatially resolved. In addition to these shortcomings, even though optical fibre optodes have diameters in the submicron range, typical penetration to the point of the nucleus would be >1% of the cell volume [5]. This level of penetration could induce severe perturbations, limiting cellular viability. Conversely, fluorescent probes, while being easier to use and offering spatially resolved images, still perturb the cell. There are a number of cytotoxic issues associated with fluorescent probes, as well as variations in signal intensity caused by non-specific protein binding, and organelle sequestration or efflux of the dye by membrane proteins. As well as providing limited experimental time once the fluorophore is loaded in the cell, fluorescent probes often require a great deal of calibration for individual cell types.

PEBBLE is a generic term to describe nanofabrication techniques that utilize biologically inert polymers to manufacture nanometre-sized spherical optical sensing devices capable of in vitro measurements. PEBBLEs are specifically designed to be minimally invasive, facilitating analyte monitoring in viable single cells or cell cultures without perturbing normal cellular functions. PEBBLEs use the same highly selective fluorescent probes normally used in biological monitoring. For example, probes such as the Alexa Fluor® series of dyes or Oregon Green are often used as reference dyes, paired with an active fluorescent probe for analyte recognition such as fluo-4 for calcium or Ru[dp(SO3Na)2]3, [ruthenium(II) tris(4,7-diphenyl-1,10-phenanthroline disulfonic acid) disodium salt] for oxygen. The paired probes are encased in a permeable biologically inert matrix, which can be thought of as a sensing platform. The reference fluorophore is chosen specifically to complement the analyte-sensitive probe to ensure ratiometric analysis can be achieved in either the excitation or emission modes.

The sensor platform offers a number of advantages; it can provide a convenient cage that facilitates the entrapment of a number of sensing components, thus offering the ability to fabricate ratiometric sensors from complementary probes or a means to achieve complex sensing schemes with the entrapment of enzymes or ionophores. The sensing components are held in a permeable nano-environment, separate from the cellular environment, which offers access only to small analytes while physically preventing interactions of larger proteins with the fluorescent probes. Ratiometric sensors are capable of reducing signal variations caused by changes in temperature, focal change shifts or photobleaching. In addition, the fabrication process confers homogeneous distribution of the entrapped dyes within the sensors allowing spatial resolution and continuity to ratiometric measurements in vitro [6].
The incorporation of nanosensors into the cell is highly dependent on matching the introduction method used with the specific cell system being investigated, as it is important to retain cellular viability. Sensor loading is another key issue as it is essential to load a suitable amount of sensors into the cell to ensure an adequate fluorescent signal. Sensor loading becomes a critical issue during long-term experimentation where the cells may still be dividing, as this will effectively reduce the amount of sensors within the cell at each division.

A variety of methods to introduce PEBBLEs to a range of cell types have been used, including functionalization with CPPs (cell-penetrating peptides), endocytosis, commercial lipid transfection agents, cytochalasin D, picoinjection, and Gene gun bombardment. These methods will be discussed in greater detail under their individual headings. All data presented relate exclusively to PEBBLEs with a polyacrylamide matrix.

CPP functionalized sensors

With PEBBLEs, the sensor matrix can be thought of as a generic platform that can be modified externally to make the sensors more acceptable to the cell. Coating a sensor with cationic peptides is one such stealth approach that has been used to incorporate sensors into cell cultures. Sensors fabricated for surface functionalization use an acrylamide matrix containing N-(3-aminopropyl)-acrylamide as one of the monomers. This monomer supplies stable NH2 groups to the outer sensor surface that can be used to attach a range of different cross-linkers, e.g. sulfo-GMBS (N-γ-maleimidobutyryloxy)sulfosuccinimide ester), without the need to chemically activate the surface of the sensor, which could be detrimental to the entrapped sensing components. These sensors can then be attached to a CPP such as one based on the Tat sequence, an amino acid sequence found in HIV [7].

A common feature of all CPPs is their cationic nature, which promotes strong interactions with the cell membrane [8]. CPPs have been used to deliver a range of cargos including proteins [9], drugs [10] and nanoparticles [11] into the cell with no discernible cellular effects or toxicity issues [12]. The definitive mechanism of CPP uptake is unknown but is thought to include ionic interactions at the cell surface and a significant endocytotic contribution [13,14]. This delivery method has been used extensively in a range of cell systems; Figure 1 (left and middle panels) shows a confocal image of hMSCs (human mesenchymal stem cells) after a 3 h incubation with Tat-conjugated nanosensors containing rhodamine B and counter stained with mouse anti-human CD105:FITC antibody, while Tat-conjugated oxygen nanosensors can be seen in a murine blastocyst in Figure 1 (right panel).

PEBBLEs, surface functionalized with CPPs, have been shown to cross the cell membrane and gain intracellular access via a simple incubation with nanosensors in cell culture. CHO (Chinese-hamster ovary) cells [15] and pre-implantation embryos have had nanosensors successfully introduced by this method with good results, adequate loading and no discernible cellular changes, making this a viable method of introduction.

Pinocytosis and phagocytosis

Pinocytosis describes the way in which small extracellular constituents are taken in by a cell, enabling the absorption of extracellular fluids and proteins in a non-specific manner. Phagocytosis is the process by which some cells ingest large objects such as cellular debris, or foreign materials such as virus or bacteria, by creating a phagocytic vacuole around the object and internalizing it. Phagocytes, in particular rat alveolar macrophages, were used successfully by Kopelman’s group [16] in the early testing of PEBBLE sensors.

Pinocytosis can be used as a control method since it indicates the amount of sensors that the cell would normally take up with no treatment other than incubation with the sensors. This allows for comparison between the effectiveness of different sensor-introduction treatments and the
predisposition of the cell to take up particles. Investigations by Pemble and Kaye [17] showed that murine embryos take up macromolecules such as proteins via pinocytosis during their development from the zygote to the blastocyst. The amount taken up increased with development, being greatest at the blastocyst stage, although the rate of uptake was not linear. This observation has been investigated to see whether it could be expanded to include small particles such as nanosensors. This was achieved by supplementing the culture medium with nanosensors at differing concentrations prior to the culturing of zona-intact and zona-denuded mouse embryos (zones were removed at the two-cell stage). The embryos were incubated and monitored over a 4-day period by confocal microscopy; however, it was found that sensor loading using this method was minimal and sporadic.

Use of phagocytes has proved effective, but is limited to phagocytic cell lines only, whereas pinocytosis has not proved a viable method for sensor loading in either cell culture or embryo culture.

**Lipid transfection**

Transfection of cells using lipids is a standard procedure often used to introduce DNA and drugs to cells; however, in this instance, it was utilized as a means for incorporating nanosensors into cell and embryo models by an incubation step. There are many different types of transfection reagent commercially available, and the choice is normally dependent on the cell type to be investigated. Escort IV (Sigma) and Lipofectamine™ 2000 (Invitrogen) are widely used examples and were prepared using the manufacturer’s instructions tailored to account for the use of nanosensors rather than DNA/RNA. Murine embryos with and without zona pellucidae were incubated with lipid transfection medium containing nanosensors in a humidified incubator with 5% CO₂ at 37°C, for different time periods. It was found that with murine embryos, Lipofectamine™ 2000 was the most effective in terms of reduced toxicity; however, both agents were found to be equally effective for loading of the sensors and ease of use. Figure 2 shows a murine blastocyst after a 4 h incubation with the Lipofectamine™ 2000 containing fluorescein nanosensors.

**Cytochalasin D**

Cytochalasin D is a cell-permeable fungal toxin from *Zygosporium mansonii*, which binds to the barbed end of actin filaments inhibiting the association and dissociation of subunits and causing disruption to the actin filaments and inhibition of actin polymerization. In the embryo, cytochalasin D inhibits cytokinesis and delays the timing of compaction and cavitation [18]. This toxin has also been used as a means to collapse the blastocoel cavity of the expanded blastocyst. At total collapse, the blastocyst can then be transferred to nanosensor-laden medium and allowed to re-expand, taking in sensors from the surrounding medium. Figure 3 shows an image of a mouse blastocyst after collapse in cytochalasin D and overnight re-expansion in nanosensor-laden culture medium.

However, it was found that the sensors were unable to pass into the blastocoel cavity, as the space between the cells was too small to allow the passage of the nanosensors, even when the tight junctions were disrupted. The sensors were observed to accumulate between the cell junctions but none were detected in the cavity. It had been reported by Flemming and co-workers [19] that, once tight junctions have formed in the embryo, it is not possible to internalize fluorophores attached to 4 kDa dextran and, given the 50 nm diameter of...
the PEBBLEs, this would probably explain their inability to enter.

**Picoinjection**

Picoinjection is a technique that allows picolitres of fluid to be injected into a single cell. This technique has been used for directing drug injections to specific cell areas and for IVF (in vitro fertilization) treatment [so-called ICSI (intracytoplasmic sperm injection)]. The injection needle is produced from a pulled capillary needle, fabricated using a pipette puller and microforge [16]. This procedure has been used successfully to inject a suspension of PEBBLE sensors into embryos at several stages prior to the hatching stage. Figure 4 shows murine embryos that had received oxygen nanosensors by picoinjection at the single-cell stage and were subsequently cultured to the four-cell (left panel) and eight-cell (overlay images, right panel) stage respectively. Nanosensors were detected in three blastomeres in the four-cell embryo and five blastomeres in the eight-cell embryo, indicating an uneven distribution of the sensors within the blastomeres prior to cell division.

The two main drawbacks of picoinjection are the high skills required to place the sensors into the cell and the low throughput nature of single-cell injection. It must also be remembered that the cells will continue to divide; however, as the amount of sensors remains constant, the result is that the sensor concentration can be halved with each successive cell division. It was found that the loading level of the sensors showed no improvement over lipid transfection or the CPP attachment; however picoinjection does offer some capacity to position the sensors within the cell and also within embryos once tight junctions have formed.

**Gene gun**

The application of gene transfer by particle bombardment was first described by Sanford and co-workers [20] and has proved to be an efficient method to transform many different organisms. The Gene gun has been used primarily as a device to transfect cell cultures with DNA or plasmids [21–23] and more recently as a means of immunization and immune system boosting [24–26].

The Gene gun has been used as a delivery method to transfer sensors into the cell. It is basically a ‘shotgun’ approach, where a blast of helium shoots dried PEBBLEs off a disk or from a prepared bullet, propelling them into the cell culture dish and embedding them randomly into adherent cells. While there is no control over the positioning of the sensors, once the correct pressure and particle concentrations are found, this method is very effective for the delivery of many sensors in a single blast with a fairly even spread of sensors throughout the cell [25,27]. Cell viability has been claimed to be excellent using Gene gun as a delivery method: 98% compared with the control [28]. Rat cerebral astrocytes can be seen in Figure 5 after Gene gun bombardment with oxygen sensors containing the oxygen-sensitive fluorophore Ru[dpp(SO$_3$Na)$_2$] and reference dye Oregon Green. The astrocytes were then exposed to hypoxic, normoxic and anoxic conditions while the change in signal from the nanosensors was monitored fluorescently.
Figure 5 | The confocal image depicts the fluorescence seen in rat cerebral astrocytes after oxygen sensors containing the oxygen-sensitive fluorophore Ru[dpd(SO$_3$Na)$_2$] and reference dye Oregon Green were introduced by Gene gun bombardment. The image displays the red and green channel at the top of the picture, relating to Oregon Green and Ru[dpd(SO$_3$Na)$_2$]; the bottom panel shows the phase contrast and overlaid images.

We have found this technique offers a high level of sensor loading, although it is only suitable for adherent cell cultures as the media must be removed prior to sensor bombardment. The handheld Gene gun has been used to insert a range of nanosensors into many different adherent cell types including astrocytes, hepatocytes, CHO cells and several epithelial cell lines [29], all with good to excellent results which showed high sensor loading and good cell viability.

Conclusions

Methods of sensor insertion can be specific to the cell type used for experimentation and many factors play a role in the choice, most importantly whether the cells in culture are adherent. The Gene gun offers high loading of PEBBLE sensors for adherent cells, while CPP functionalization has proved effective for cell cultures generally with varying levels of sensor loading, depending on the cell type. Lipid transfection gives good results once concentration of transfection reagent and sensor loading are established. Picoinjection allows location specific PEBBLE delivery, although the loading is not as high as with other methods and this technique is therefore more suited to larger cell types where single-cell investigations are of interest. Pinocytosis and cytochalasin D are not suitable for pre-implantation embryos once the tight junctions have formed, since there is a finite size of molecule able to enter. It is clear from the work reported that both the method of delivery and cell type of interest are critical to achieve optimal intracellular delivery of nanosensors.

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