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Review

Cell preservation methods and its application to studying rare disease

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ABSTRACT

The ability to preserve and transport human cells in a stable medium over long distances is critical to collaborative efforts and the advancement of knowledge in the study of human disease. This is particularly important in the study of rare diseases. Recently, advancements in the understanding of renal ciliopathies has been achieved via the use of patient urine-derived cells (UDCs). However, the traditional method of cryopreservation, although considered as the gold standard, can result in decreased sample viability of many cell types, including UDCs. Delays in transportation can have devastating effects upon the viability of samples, and may even result in complete destruction of cells following evaporation of dry ice or liquid nitrogen, leaving samples in cryoprotective agents, which are cytotoxic at room temperature. The loss of any patient sample in this manner is detrimental to research, however it is even more so when samples are from patients with a rare disease. In order to overcome the associated limitations of traditional practices, new methods of preservation and shipment, including cell encapsulation within hydrogels, and transport in specialised devices are continually being investigated. Here we summarise and compare traditional methods with emerging novel alternatives for the preservation and shipment of cells, and consider the effectiveness of such methods for use with UDCs to further enable the study and understanding of kidney diseases.

1. Introduction

The advancement of science depends on international collaboration and sharing of resources, including cellular and tissue samples. Acquisition of cells is relatively simple, due to the abundance of commercial cell lines available from globally established companies. Typically, these commercially available cells are either derived from tumours, or cells are artificially stimulated to proliferate continuously resulting in an immortalised cell line [1]. Alternative sources of cell and tissue samples include non-profit organisations (i.e., biobanks, biorepositories and cell banks) and primary cell collections (i.e.: patient tissues and liquid biopsies).

The investigation of rare human diseases are particularly reliant on global collaboration, often for sourcing patient primary cells and tissues. The studying of disease mechanism and the design of therapeutics are often reliant upon cellular models and sharing these precious resources in a collaborative way speeds up vital research. The ability to ship cells safely for research purposes therefore is key to this often global collaborative effort. It is therefore vital that a high proportion of cells survive the entirety of the transportation process and are viable for downstream applications in their destination laboratory. Typically, biological samples, after freezing, are shipped under cryogenic conditions, preventing biological activity and sample degradation during transit.

Ciliopathy syndromes are an example of a group of rare genetic diseases that are frequently studied using primary cells, requiring long-haul shipment between laboratories. Primary cilia are found on the surface of almost all cells and are finger-like protrusions involved in key cellular signalling pathways including the Sonic hedgehog (Shh) and Wingless (Wnt) pathways [2]. In humans, ciliopathies can affect a number of organ systems including the brain, eyes, liver, lungs and kidneys, with the latter often resulting in end stage kidney disease. Ciliopathies affecting the kidneys have recently been studied using cells derived from patient urine [3]. An average of 2000–7000 renal cells are shed in urine on a daily basis [3] which under the right conditions can be collected, cultured and used for mechanistic studies and other

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applications. We have successfully used urine derived cells (UDCs) to further our understanding of renal ciliopathies such as Joubert Syndrome (JBTS) [4–6] without the need for an invasive native kidney biopsy.

The collection of urine for renal cell isolation dates back to 1972, when Sutherland and Bain successfully cultured exfoliated cells found in the urine of new-born children [7]. Since then, the practice has continued to gain traction, with UDCs used for the study of a variety of diseases [8–13]. Interestingly, there are thought to be two main sub-populations of UDCs, known as Type I or Type II and their classification is determined morphologically [8]. Currently, those cells with a 'rice-like' shape are classified as Type I, whilst those with a 'spindle--shape' are considered as Type II [8]. Furthermore, it has also been argued that there is a third sub-population of UDC, known as urine-derived epithelial cells [8]. Multiple studies have concluded that UDCs express surface markers which are more commonly associated with stem cells. Examples of such markers include the haemopoietic markers CD73 and CD44 [11,14–20], and more interestingly the embryonic stem cell marker SSEA4 [9,11,13,14,17,18,21]. These findings suggest that the relative plasticity of UDCs allows for not only the study of renal diseases but multiple others including cardiac [22] and skeletal [18] conditions.

Certain diseases, including JBTS and other related inherited conditions, have increased prevalence in specific geographical populations, making cross institutional collaboration or travelling to patients vital to research. It is important to note that a short turnaround of 4 h transported on ice, is accepted as the gold standard for isolating human cells capable of expansion from human urine samples to decrease cellular activity and protect viability [23]. Typically, researchers will visit laboratories close to patients, to isolate, culture and then freeze down cells before their shipment back to home institutions. Understandably, isolating and culturing UDCs in this manner can be a timely and costly progress. Furthermore, recovery of these cells can be somewhat limited following preservation and transport under cryogenic conditions, highlighting the urgency for exploration and validation of other shipment methods to give increased numbers of viable UDCs.

This review will focus on current and emerging methods deployed for the long-haul shipment of cells for use in scientific discovery and research. Comparisons will be drawn between a number of different systems, including the current cryogenic shipments, the use of novel hydrogel-based encapsulations, and modified tissue culture plastics to explore their respective potential for use with UDCs to study ciliopathies.

2. Thermal preservations and shipment methods

2.1. Cryogenic preservation

Typically, mammalian cell lines are frozen down, and stored in sealed cryogenic vials prior to long distance transport at temperatures below 0 °C, known as cryogenic shipment. It is quick and easy to freeze flasks of cells down, into cryovials, which are small and easy to transport. Cells transported on dry ice (DI) and liquid nitrogen (LN) are stored in 10% dimethyl sulphate (DMSO), a cryoprotectant, which at ambient temperatures is proven to be cytotoxic to cells [24]. A study using neural stem cells found that in the slow freezing process of cells, DMSO concentration is often influential on the level of cellular recovery and viability [25]. During the slow freezing process, cells are gradually dehydrated to prevent the formation of ice crystals from intracellular water [26]. These protective properties are maintained as long as samples remain frozen. However, after 4 days DI can evaporate, resulting in sample thawing and cells sitting in DMSO. A study involving human cell lines including RPE, osteosarcoma (U2OS) and cervical cancer (HeLa) [27] unsurprisingly observed poor cell recovery and viability following this described thawing. Furthermore, the cryopreservation of cells has shown to decrease cellular viability following the thawing procedure. One study focussing on human primary cell lines found that severe hypothermic treatment significantly reduces both cellular recovery and viability [28]. In fact, the best viability was found in those cells which had been stored and shipped between 16 °C and 22 °C [28] suggesting that a new 'gold standard' of shipment is required.

Interestingly, the effect of cryogenic preservation in different species, has been demonstrated to have variable effects upon cellular activity. Following freeze-drying of spermatozoa from a number of species, including rodents, primates [29] and humans [30] interspecies variations in sperm viability and motility were observed. In human sperm samples it was found that DNA integrity was maintained in 80.6 ± 11.3% of samples [30]. In comparison, another study in rhesus monkeys found that between 25.3% and 43.7% of samples had DNA damage [31] following cryopreservation. Despite cryopreservation being deemed the gold standard, these studies highlight the importance to consider interspecies differences when attempting to use new methods of preservation in the future.

In relation to the study of ciliopathies using primary cells extracted from urine, a limited cellular recovery and viability suggest that this traditional shipment method is not well suited here. Further additional limitations, such as evaporation of dry ice, potential risk to courier staff, and the prohibition of DI transport [29], are further evidence as to why there are demands for new methods of transporting cell and tissue samples.

2.2. Hypothermic preservation

A short-term alternative to cryopreservation, hypothermic storage involves the maintenance of cells between 4 °C and 10 °C. The benefits of this method of preservation include a reduction in metabolism, degradation in energy storage and reduced oxygen demand [30]. Currently, UDCs are transferred from the hospital / clinic to the laboratory on ice, within 4 h, and therefore can be considered to be transported via hypothermic preservation. However, improper hypothermic storage conditions can result in irreversible structural changes in cells resulting in cellular apoptosis [31]. Furthermore, osmotic swelling (due to disruption of ion balance), oxidative stress and variation in both intra- and extracellular pH levels can also arise during hypothermic storage [32].

In 2013, the recovery of UDCs preserved in urine at 4 °C for 24 h was investigated [33]. Around 73% of cells stored in their respective whole urine, for a maximum of 24 h at 4 °C, were identified to be viable, 10% fewer than those from fresh urine samples [33]. Overall, it took longer for UDC colony development from samples stored at 4 °C compared to freshly collected samples [33]. Notably, both groups of UDCs were reported to share the same cell surface markers, indicating that this prolonged storage of cells at 4 °C did not affect UDC characteristics [33]. These findings suggest, that in the case of domestic transportation of samples, a maximum of 24 h will allow for the isolation and culturing of adequate numbers of UDCs. However, caution should still be taken, as delays in transport may extend this period, risking the viability and integrity of samples upon receipt. It is for this reason, that it is advisable to continue to explore alternative methods of shipment for these precious UDCs.

2.3. Normothermic preservation

Unlike cryogenic and hypothermic preservation, normothermic preservation involves storing cells, organs or tissues around 37 °C, with success reported in murine liver samples [34] as well as *ex vivo* kidney perfusion renal studies [35]. Although predominantly associated with the preservation and shipment of whole organs intended for transplantation, a lot can be learned about the effect of normothermic preservation on kidney cells and tissues.

When applied to a cellular setting, the transport of samples in tissue culture flasks can somewhat be considered as a form of normothermic

preservation. The idea here is that culture medium will prevent the displacement of air and ultimately protect cells. However, rapid depletion of oxygen resulting in pH changes has limited transport in this manner to a maximum of 24 h [36]. In relation to the transportation of UDCs this 24 h window could be successful in the transport of domestic samples, however international shipments may prove to be more complicated using this method. Furthermore, agitation occurring during transfer also poses an issue, with the possibility for detachment of cells in monolayers [36]. Therefore, the transport of UDCs in this manner would be inappropriate, as it is typical to obtain low densities of cells during early stages of culture. Perhaps a resolution for this limitation would be to send confluent flasks of UDCs, however the cell identity of UDCs would be questionable, due to aging cells adopting a more fibroblastic like morphology. Additionally, strict air-regulations preventing the shipment of liquid samples also hinders the widespread use of this shipment method [27], again posing a limitation for the use of the long-haul shipment of UDCs.

3. Specialised transport devices

3.1. Transwell inserts

A transwell insert is a type of tissue culture plate accessory, containing a semi-permeable membrane, and can be placed inside of traditional multiwell tissue culture plates. The inserts are designed to hang into wells, leaving a clearance of around 1 mm from the base. They can be deployed to study cells in indirect coculture, anchorage dependant/independent cell lines, and cells in microfluidic systems [43], with one of the earliest studies conducted in 1953 [44]. In 2017, Miller et al. created a novel transport system based upon the transwell insert principle [41]. In regards to cell preservation and shipment, the idea was to maintain cell viability without needing hypothermic preservative measures [41]. The group investigated the cellular viability of three human cell lines including Colorectal Adenocarcinoma (Caco2), Lung Adenocarcinoma Epithelial (A549) and Hepatocellular Carcinoma (HepG2C3A) after 48 h mimicking shipment in their devices. In brief, the novel transport system consisted of a transwell carrier made of polycarbonate sheets [41]. Threaded polycarbonate sealing lids were modelled on those used with cryovials, and a viton o-ring added to ensure an airtight fit to protect the contents [41]. Devices were designed to allow a clearance of 3 mm from well bases, and were compatible with 6 and 24 well plates [41]. Following analysis via live and dead staining, the group found no significant difference between the cell viability of confluent Caco2, A549 and HepG2C3A cells shipped in their device compared to controls [41]. The device was also successful maintaining the viability of sub-confluent cultures of Caco2 and HepG2C3A with a recovery rate of around 70% in those samples which had underwent artificial shipment conditions [41]. These findings are exciting, as they offer the chance to transport cells at ambient temperatures whilst maintaining the integrity of established cultures. However, as demonstrated by the sub-confluent A549 population, the device is not suitable for every cell type, as only 22% of cells were deemed viable following transport [41]. It is for this reason it is fair to conclude that the use of adapted transwell insert may not be suitable in UDC transport, with low cellular concentrations often reported following immediate sample collection.

3.2. Filtration and storage devices

It is well known that a heterogenous cellular population exists in urine, including epithelial [4,6] and squamous cells, as well as macrophages. These UDCs can then be used in the study of multiple patient specific diseases including ciliopathies like JBTS [4] or as diagnostic tools of bladder cancer [42,45]. Established isolation protocols currently still require the heavy involvement of specialist laboratory skills and equipment, thus limiting collection of some rare samples. In order to overcome this, in 2015 a novel urine cell collection device was investigated to improve the cell yield at collection and sample transportation to enable for the effective collection of DNA as a diagnostic tool for bladder cancer [42]. Initially urine samples (between 100 and 400 ml) or cultured cells were voided and passed through the novel filtration device, with a pore size of 8 μm [42]. Following this filtration, samples presumed to contain UDCs were then stored in a cassette containing a lysis/stabilization buffer which was released upon the application of the device lid [42]. This air tight seal then allowed for the secure transportation of UDCs which were stored at room temperature. A recovery of 70% was found in samples which contained 1000 cell/100 ml of urine [42]. Interestingly, a lower rate of recovery was observed in those samples with an increased cell density ($<5\%$ in 5×10^6/100 ml of urine) presumably due to saturation of the device filter [42]. Furthermore, it was concluded that the buffer used during the shipment process was effective in maintaining the stability of DNA [42]. The improved capture of cells, along with the preservation of DNA, from urine samples with low cell densities suggests that similar technology may prove beneficial in the isolation of UDCs, as it is common to observe low cellular yields of this cell population. However, it is important to note that filtration success is heavily dependent upon a number of factors including; cell characteristics (size and deformative capability); filtration parameters (surface pressure across the filter and flow rates) [46]; and filter properties (pore size, quantity and distribution) [46]. Therefore, in order to successfully use such a filtration device for the isolation, preservation and transport of UDCs, a cell line specific optimisation is undoubtably key to success here.

4. Gel shipments

The idea of encapsulating live cells into a synthetic gel is not new, in fact the earliest reported attempt was by Bisceglie in 1934, who described successful encapsulation of cancerous cells into hollow polymer structures [47]. Since then, this idea has been stretched and built upon, with a number of attempts, varying in their degrees of success, made to encapsulate cells within gels as a method for transporting viable cells. In 2007, live mice fibroblasts, cell line L929, were successfully encapsulated in and released from a water-soluble phospholipid polymer-based hydrogel following storage between 10 °C and 24 °C [48]. Released cells were shown to have high activity, with hydrogels shown to be porous in nature allowing for diffusion of bioactive molecules [48]. A limitation of the water-soluble phospholipid polymer however, is that when a temperature of 37 °C is reached, the gel melts [48] thus posing a threat during transport where increased temperatures are experienced. Since these early studies, the prospect of encapsulating, mixing or sealing human cell lines with different basal gel compositions has gained momentum, offering exciting new approaches to overcome limitations associated with cryogenic and other ambient methods of cell storage and shipment (see Table 1).

4.1. Agarose gels

One of the earliest ambient gel-based methods of cellular transport was tested in 2009. Madin Darby Canine Kidney (MDCK), Human Embryonic Kidney *293* (HEK-293), and A549 cells were transported in plates and dishes coated with an agarose gel [49]. Agarose allows for the supplementation of both nutrients and moisture to cells, enabling increased cell viability. Cells were cultured in plates, before the removal of media and replacement with a 1% agarose-media mixture used to coat cellular monolayers. Successful culturing of MDCK cells was reported, following 3 days storage at room temperature [49]. Morphology of cells following agarose-medium removal was also found to be normal, suggesting that this cellular sealing process is successful in the maintenance of cells at ambient temperatures. Unsurprisingly, the study also found that following the peeling removal of the solidified agarose-media gel, a number of cells were removed due to their attachment to the solidified

Table 1
Various methods of transporting cells and their associated advantages and disadvantages.

Preservation Method	Advantages	Disadvantages	Cell Lines/Tissue Investigated	Viability Assessment	Findings
Dry Ice	- Current widespread use - Maintenance of high levels of cell viability	- Thawing of dry ice leaving cells in DMSO - Expensive - Air travel prohibited in multiple countries	- Rat liver tissue [37]	- Protein and enzyme analysis [37]	- Sufficient organelle integrity remains following freeze thawing [37]
Liquid Nitrogen	- Current widespread use - Good levels of cell recovery	- Many couriers refuse to handle liquid nitrogen	- Rat liver tissue [37] - Peripheral Blood Mononuclear Cells [38]	- Protein and enzyme analysis [37] -Trypan blue dye exclusion [38]	- Sufficient organelle integrity remains following freeze thawing [37] - ≤75% viability in cryopreserved samples [38]
Hypothermic Preservation	- Combination with extra-cellular-matrix micropar-ticles increases cell viability	- Short term storage solution - Increased cellular apoptosis in incorrect storage	- GLC-82 and MCF-10 [39]	- Live/Dead Viability/ Cytotoxicity Kit, Molecular Probes [39][40]	- Decreased cellular viability over time in hypothermic preservation methods. Improved viability observed with the use of microparticles [39]
Tissue Culture Flask/Specimen Collection Tube	- Transport cells which are sensitive to cryopreservation	- Limited to 24 h transit period - Couriers refuse to handle liquids - Agitation during transit causes mechanical detachment of cells	- Lymphocyte [36]	- Lymphocyte Proliferation Assay (LPA) [36]	- Increased levels of LPA in fresh samples compared to those shipped in specimen tubes [36]
Bodily Fluids	- Recovery of up to 73% of cells reported - Transported as sample as collected - No need for specialist laboratory skills or equipment	- Limited to 24 h transit period - Couriers refuse to handle liquids	- Urine derived cells [33]	- Trypan blue dye exclusion [33]	- 13.65 ± 4.96% (ratio of live cells to total cells) following 24 h preservation in urine [33]
Transwell Inserts	- Recovery rate of around 70%	- Limited to 48 h transit period - Not compatible with all cell lines	- Caco2 - A549 - HepG2C3A [41]	- Live/Dead staining (Invitrogen #L3224) [41]	- No significant difference found in cell viability of control cells and 'shipped' cells [41]
Filtration Devices	- Easy sample collection at home or in hospital - Large collection reservoir (500 ml)	- Filter saturation decreases viability of cells	- Urinary cells [42]	- Quantified number of cells captured using droplet digital PCR (ddPCR) to identify the total number of mutant *FGFR3* molecules [42]	- Lowest cellular concentrations had cell recovery rates of 70% compared to 5% in those with higher cellular concentrations [42]

material. For transport via this method, temperatures of between 1 °C and 37 °C are preferable to prevent cellular damage [49]. When considering if this method would be suitable for use with UDCs, it is fair to suggest that the loss of cells following agarose-media removal is concerning; UDCs tend to form colonies with a low cell density. Therefore, it is vital to ensure that the maximum number of cells survive all aspects of the transport procedure, suggesting that other forms of gel-based transport systems may prove more beneficial for use with UDCs.

More recently, the recovery of myoblasts, a primary human cell line, have been investigated following 3 days transportation at a temperature of between 20 and 22 °C over 3 days [27]. It was discovered, that those cells which were resuspended in a 1:1 ratio of complete Dulbecco's Modified Eagle's Medium (DMEM) to a transporter solution were successfully recovered [27]. Transporter solution consisted of 2% low melting point agarose supplemented with 20 mM filtered sterilised HEPES, pH 7.4; this solution was then kept at 37 °C before the addition of cells [27]. This apparent success in the preservation of primary cells suggests that there may be potential benefits in using this ambient shipment method with UDCs in future investigations, without risking the quantity of viable cells received following the release process.

4.2. Matrigel

Matrigel is a gelatinous mixture consisting of proteins (including laminin, entacin, collagen and proteoglycan), and is secreted from Engelbreth-Holm-Swarm (EHS) murine sarcoma cells often used to promote the survival of numerous cell types [50]. Historically, Matrigel has been used in the development of three-dimensional (3D) cultures like spheroids, as well as investigating cellular invasion. The material is flexible in storage temperature, capable of being frozen at −20 °C, existing in a liquid state at 4 °C and solidifying at 10 °C. These attractive properties have allowed for investigations in the transportation of a number of human cell lines using Matrigel as a semi-solid vessel at ambient temperatures [29]. Surprisingly, human breast cancer cells (MCF-7) were found to have good viability following 7 days storage at room temperature when mixed with Matrigel [29]. An optimal concentration ranging between 1×10^6 and 5×10^6 cells per ml of Matrigel was found to have the best levels of survival, as well as maintaining normal morphology when compared to cells which had been thawed from liquid nitrogen [29]. To further validate these findings, the group transported MCF-7 cells, mixed with Matrigel to various different locations, all of which had a 4-day transit period; recovery of up to 90% of cells was reported [29]. Similar success was reported in additional human cell lines including A549, U2OS, and breast cancers (MDA-MB231 and SUM159) [29]. It is important to note, that the cell lines used in this investigation are all of a cancerous heritage, and therefore translation to UDCs may not be as successful, due to the increased level of sensitivity associated with primary cell lines. In comparison, cancer cells are known to have high proliferative activity and aberrant metabolism [51] both of which can be considered as factors assisting in cell survival following stress, like that seen in cell

shipment.

In respect to primary cell lines, interestingly, Matrigel containing the ROCK inhibitor Y-27632, has shown to improve the recovery of human pluripotent stem cells (hPSCs) following cryopreservation [52]. Furthermore, in relation to UDCs, treatment with Y-27632 has also been shown to increase the efficiency of UDC isolation, proliferation and differentiation [52]. ROCK inhibitors have previously been shown to promote proliferation and viability of cells, as well as enabling the differentiation of MSCs [52–54]. Collectively, these findings imply that the use of Matrigel may assist in the preservation of key cellular activities following the release of UDCs from the matrix after shipment. However, during shipment, increased capability for cell differentiation can be deemed a limitation, as preferably these primary UDCs would be received in an undifferentiated state allowing for flexibility in experiments.

5. Hydrogels

Hydrogels are a class of biomaterial boasting good levels of biocompatibility and biodegradation. Cells can be encapsulated within the hydrogel 3D structure allowing for increased diffusion of nutrients and metabolic waste preventing cellular damage [55]. There are three major classifications of hydrogel system described, natural polymer, synthetic polymer and supramolecular hydrogels (Table 2), with structural properties including porosity and pore size [56] also linked to the materials biocompatibility potential.

Typically, hydrogels are fabricated using the crosslinking of polymers, which along with a richly hydrated matrix allow for the mimicking of the extracellular matrix (ECM) (Fig. 1) resulting in improved biocompatibility [56]. The ECM is essential in dictating the differentiation of stem cells, with hydrogels considered to offer artificial physiochemical stimulation similar to that which naturally occurs in tissues [57,58]. Furthermore, physical signals can also be transferred via the ECM, travelling through the cytoskeleton of cells before conversion into biochemical signals responsible for the activation or inhibition of proliferation, migration and differentiation [59]. Cellular adhesion, proliferation and viability of encapsulated cells is very much dependent upon cell-matrix interactions [60], with the hydrogels synthetic ECM shown to assist in cellular adhesion and spreading [61].

In regards to urine derived stem cells (USCs), when seeded onto hydrogel scaffolds, have shown good levels of viability, as well as improved capabilities within tissue engineering [12,62]. These findings imply that hydrogels offer good levels of biocompatibility for use with urine derived cells, offering promise for use in long distance transportation of this sensitive cell type. Recently, there has been increased interest into the implementation of hydrogels in cryopreservation systems as reduced ice crystal growth is observed [63–66].

5.1. Natural polymer hydrogels for cell encapsulation: collagen, chitosan and gelatin

Of all the natural polymer hydrogels investigated for their cellular encapsulation capacity, collagen is by far the most commonly studied. High levels of collagen are found in a number of ECMs, including that of skin, bone, ligament, muscle tendon and blood vessels [87]. There are multiple types of collagen, with Type 1 accounting for 90% of protein in human connective tissues, and therefore the most commonly used in hydrogels [88]. Typically collagen-based hydrogels, due to their increased stiffness, are predominantly used in tissue engineering, and are especially popular in cartilage regeneration applications as well as drug delivery vehicles [75–77]. To date, there is a lack in the description of collagen-based hydrogels implemented as cell shipment models, however, the successful cell encapsulation of fibroblasts and adipose derived stem cells in a Type I atelocollagen-based hydrogel has been described [79]. A 4-min thermal shock release process (at 48 °C) was found to be the most successful method of extracting cells, with the least impact upon both cell viability and proliferation [79]. These findings suggest that there is potential for the use of collagen-based hydrogels for shipment of cells, with a maximum of a 72 h encapsulation period before reduction in viability starts to occur [79].

Gelatin is a biocompatible polymer and a product of thermal degradation of collagen. It is stable at 37 °C and solidifies under cooling temperatures to give a gel [80]. In 2014, a basic gelatin-based hydrogel was fabricated to allow for the encapsulation of murine embryonic fibroblasts, cell line NIH-3T3 [80]. Cells were covered with a G-media mixture at 37 °C before gelation at 10 °C and 23 °C; after 2- or 7-days storage under transport conditions, cells were released from the gelatin-media mixture at 37 °C [80]. An increased preservative capacity was shown in those NIH-3T3 cells which were stored in the G-media gels across all test groups [80]. However, like Matrigel based systems, the fact that solidified gels melt once they reach 37 °C can prove difficult during transport, risking cellular viability if exposed to increased temperatures for prolonged periods. More recently, studies involving hydrogels consisting of Gelatin-methacryloyl (GelMA) [89,90], GelMA and silk fibroin [91,92] and gelatin bioink [93] have all shown exceptional viability of encapsulated cells highlighting gelatin-based hydrogels as a suitable carrier of multiple cell types.

Another natural polymer, of a hydrophilic nature, used in the fabrication of hydrogels is chitosan. Chitosan can be derived from a number of natural sources, including the shells of crabs [94]. A derivative of chitin, chitosan is a linear polysaccharide containing randomly dispersed N-acetyl-D-glycosamine groups [95]. Chitosan also has similar

Table 2
Characteristics of the most commonly used basal compositions of hydrogels in biomedical applications. A brief summary of some of the most frequently used hydrogels in biomedical applications including cell storage, cell transport, drug delivery and regenerative medicine.

Basal Composition	Class of Hydrogel	Gelation Method	Reported uses	Cell Lines Investigated	Reference
Alginate	Natural Polymer	- Covalent bonding via the addition of ions (Ca^{2+}, Ba^{2+} or St^{2+})	- Cell encapsulation - Cell shipment - Cell storage	- Mesenchymal stem cells (MSCs) - Adipose-derived stem cells (ADSCs)	[67–71]
Chitosan	Natural Polymer	- Chemical crosslinking - Covalent bonding - Ionic bonding - Hydrogen bonding - Hydrophobic association	- Cell Scaffold - Tissue Engineering - Tissue Regeneration - Drug Delivery	- NIH-3T3 - Periodontal ligament cells (PDLCs)	[72–74]
Collagen	Natural Polymer	- Thermal polymerisation ranging between 4 °C and 37 °C	- Cartilage regeneration - Drug delivery	- Fibroblasts - ADSCs	[75–79]
Gelatin	Natural Polymer	- Wet spinning accompanied by Ca^{2+} ions	- Cell encapsulation and storage	- Murine embryonic fibroblasts (NIH-3T3)	[80–82]
Hydraulic Acid	Synthetic Polymer	- Photo-cross-linking via UV photoirradiation	- Delivery vehicles - Assist cell differentiation	- UDCs - Bovine articular chondrocytes	[83,84]
Poly (ethene-glycol)	Synthetic Polymer	- Photo-polymerisation - Michael-type addition reaction	- Cell encapsulation	- Chondrocytes - Murine pancreatic β-cells (MIN6)	[85]

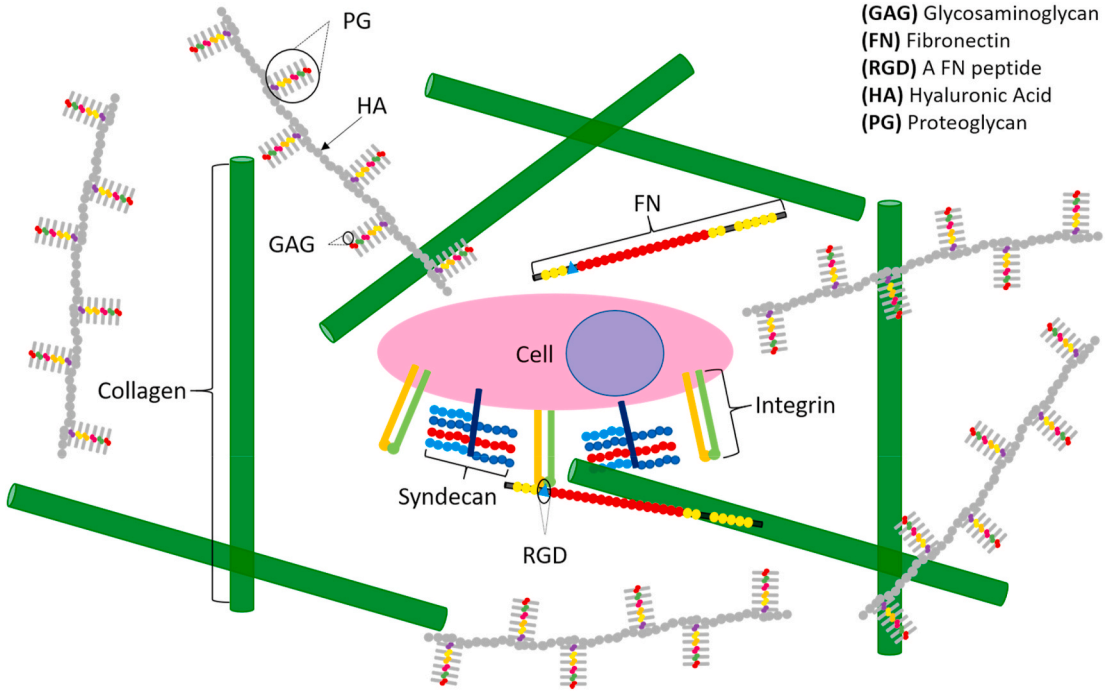

Fig. 1. Simplified structure of the extracellular matrix (ECM). Simplified diagram of the ECM and its key components. In brief, a cell is surrounded by the ECM. Cells adhere to the ECM via laminin (LN) and fibronectin (FN) examples of adhesive proteins. ECM specific proteins are found within gelatinous substances which are negatively charged and rich in polysaccharides. These substances are known as Glycans, including glycosaminoglycans (GAG) and proteoglycans (PG). Of these glycans, GAGs are arranged in a linear formation and can be either non-sulphated (Hyaluronic Acid- HA) or sulphated. Glycans are essential in the diffusion of nutrients and signalling molecules. Figure adapted from Ref. [86].

glycosaminoglycans as those found in ECM (Fig. 1), as well as good antibacterial, and biocompatible properties [96]. Previous reports have shown that NIH-3T3 cells are able to migrate into the chitosan-hydrogel matrix due to the gel's good levels of biocompatibility [72]; this cell migration can be somewhat considered as an indirect method of cellular encapsulation. In comparison, a more direct encapsulation of periodontal ligament cells (PDLCs) into a chitosan-based hydrogel [73,74] has been shown, thus removing the dependency of cell migration processes for successful cellular encapsulation. However, despite attractive biological properties, chitosan-based hydrogels, like gelatin-based gels, are easily affected by temperature, with the most rapid gelation occurring around 65 °C [97], which can affect cell viability. Therefore, chitosan tends to be reserved for tissue engineering and drug delivery applications rather than cell preservation and shipment.

5.2. Natural polymer hydrogels for cell encapsulation: alginate

One of the most commonly used natural polymers in hydrogels is alginate, a polysaccharide derived from seaweed, and is proven to be a good candidate for the delivery of cells in therapeutic applications [98,99]. Alginate is a linear block copolymer, containing a number of α-1,4-L-guluronate and β-1,4-D-mannuronate repeating monomeric units [100]. Typically, alginate hydrogels are cross-linked at room temperature with either calcium (Ca^{2+}), Barium (Ba^{2+}) or Strontium (St^{2+}) cations [69] (Fig. 2). Increased levels of interest are also focussed on the potential for alginate hydrogels and nanoparticles in the tissue engineering field, as well as their potential for use as drug delivery systems [70]. Alginate-based hydrogels have previously been deployed in the slow freezing of cells encapsulated within microcapsules to enhance the cryopreservation of mesenchymal stem cells (MSCs) and adipose-derived stem cells (ADSCs) [67,68] as well as an alternative to cryopreservation of MSCs and mouse embryonic stem cells (mESCs) [101]. Alginate based hydrogels have also been proven to successfully preserve MC3T3 osteoblasts cells (a murine cell line), with a cellular viability of between 70 and 80% found when a low molecular weight alginate was used [104]. This apparent relative interspecies compatibility explains why alginate remains an attractive material for cell preservation.

Alginate has proven itself to be beneficial in a number of preservative applications, including protecting especially sensitive biological materials during cryopreservation [102]. More recently however, alginate has been deployed in normothermic preservation of cells, with optimal recovery temperatures of 15 °C following MSC encapsulation reported [71]. The relative flexibility within varying thermal storages of cells indicate that alginate-based hydrogels offer exciting new prospects for improving the transportation, and viability, of urine derived cells.

Associated limitations of alginate-based hydrogels include both bead size and cellular density within the bead itself. An optimal cellular concentration must be established, in order to ensure that the correct levels of nutrients are contained within the bead cells are encapsulated within (Fig. 2A), whilst also ensuring that any metabolic waste products are also efficiently excreted from the bead via diffusion [103]. In regard to UDCs, the optimisation of cellular concentration may prove timely; there is also the potential risk of the loss of precious viable cells during the encapsulation process itself.

5.3. Synthetic polymer hydrogels for cell encapsulation: Poly(ethylene-glycol) and hyaluronic acid

Synthetic-based hydrogels are both biomimetic and biodegradable, capable of containing over eighty percent of their total weight in water [106]. Programmable macroscopic properties, including mechanical swelling, as well as relatively easy controllable properties like degradation, make synthetic polymer-based hydrogels especially attractive for biomedical applications [106]. *In situ* polymerisation in direct contact with live biological materials, including cells [106], mean that hydrogels present themselves as highly attractive biomaterials as alternative vessels for cellular preservation and long-haul shipment.

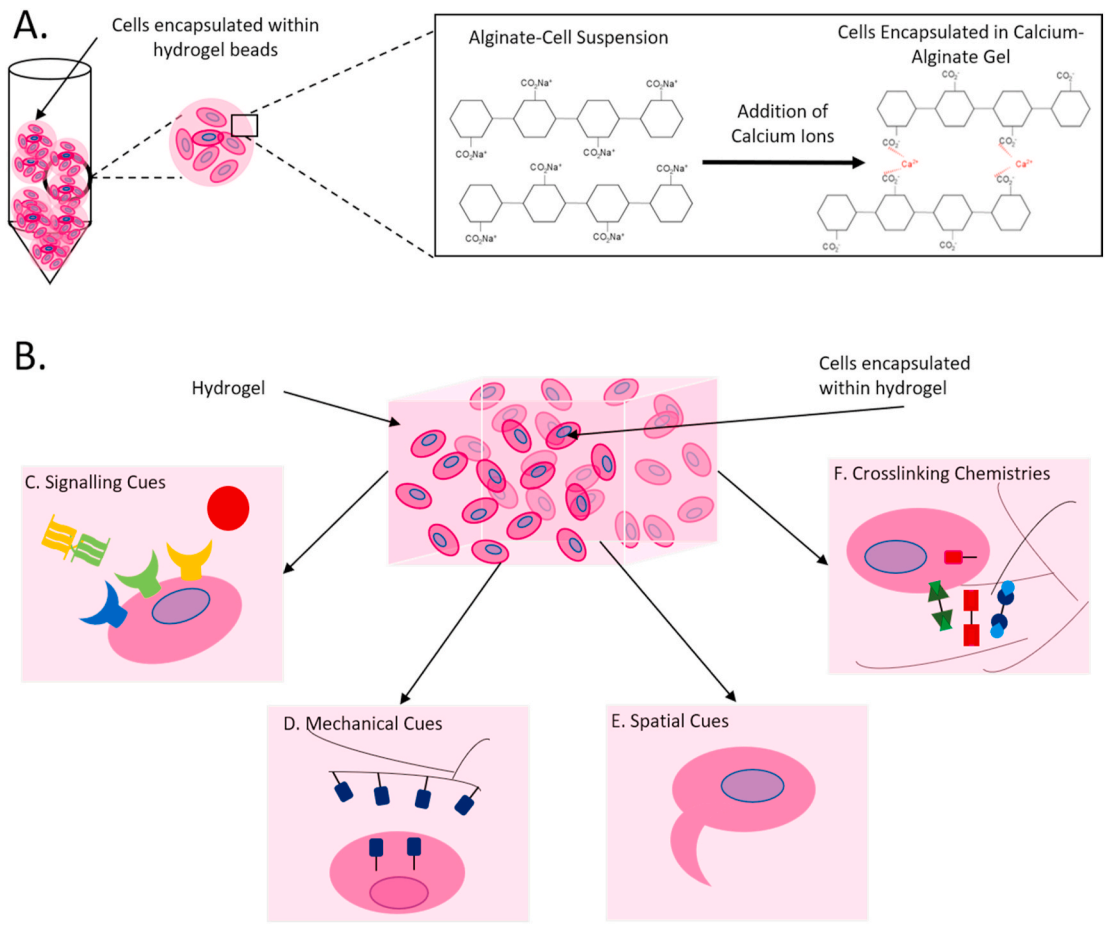

Fig. 2. Hydrogel and Cell interactions. (A) Simplified schematic of the encapsulation of cells within an alginate-based hydrogel bead system. Cells are resuspended in an alginate solution before the addition of calcium ions. Crosslinking between the alginate and calcium causes the encapsulation of cells during the gelation process, to give calcium-alginate-based hydrogel beads. The gelation process is described in Ref. [104]. **(B) Simplified examples of the cell-hydrogel-matrix interaction and specifically engineered points within a hyaluronic acid-based hydrogel.** (C) The incorporation of bioactive signals can be achieved via direct addition to the scaffold (the hydrogel in this example) or via covalent bonding to polymer. (D) Mechanical properties can be adjusted by utilizing degradable crosslinkers to regulate cellular mechanosensing. (E) Patterned bioactive signals, porosity control, and topographical patterns, collectively referred to as spatial cues are influential in cellular migration. (F) New forms of crosslinking chemistries can be engineered to covalently crosslink the hydrogel network. Figure adapted from Ref. [105].

An example of a synthetic polymer-based hydrogel is poly (ethylene-glycol) (PEG), used to encapsulate multiple cell lines including MSCs [107], chondrocytes [108] and macrophages [109]. It has been shown that cells encapsulated in PEG-based hydrogels are capable of secreting their own ECM proteins into the hydrogel matrix, which can influence both cell behaviour and fate [110]. Adhesion peptides including fibronectin are secreted as a component of ECM, allowing encapsulated cells to adhere and spread within the hydrogel matrix [107,111]. In regards to PEG-based hydrogels' potential application as a shipment method, the secretion of ECM proteins could pose a hindrance for those groups which desire UDCs to remain in an undifferentiated state. Ideally, for shipment of cells, an induced hibernating-like state would be beneficial, so as to prevent the metabolic activity of cells whilst also protecting their viability.

The hydrogel-matrix-cell interaction within PEG-based hydrogels can be influenced via adhesive peptide sequences [85]. In fact, murine pancreatic β-cells (cell line MIN6), when encapsulated in unmodified-PEG hydrogels exhibited no cell-cell or cell-matrix interactions [85]. After 10 days in culture in unmodified PEG hydrogels, a reduction in the viability of MIN6 was observed [85], suggesting that in order to be considered as feasible shipment vesicles, hydrogels must be capable of providing cell-matrix interactions. However, unlike natural polymer-based hydrogels, with synthetic compositions, like PEG, it is often difficult to remove unpolymerized monomer residues, which are cytotoxic in biomedical applications [82].

Hyaluronic acid (HA) is composed of a number of repeating disaccharide units, including D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc), arranged to form a high-molecular-mass glycosaminoglycan [84]. HA has been identified as a major component within the ECM of mammalian connective tissues, thus explaining why HA is a suitable candidate for implementation into synthetic hydrogels mimicking the ECM. In 2008, viable bovine articular chondrocytes were successfully loaded into HA-based hydrogel beads, via injection using glass capillary microneedles (diameter of 248 ± 34 μm) [84]. The study found that the cell seeding efficiency was low due to large volumes of cells escaping from the bead caused by hydrostatic pressure during cell suspension into the HA-water filled network [84]. Despite this low encapsulation efficiency, an enhanced cell growth and proliferation rate was observed suggesting that encapsulation was effective in maintaining cellular viability. Interestingly, in 2011 another study showed that fibroblasts, endothelial and mesenchymal stem cells can all successfully be encapsulated and display high levels of cell viability after 7 days within HA-based hydrogels, without adhesion and therefore interactions between the HA-Cell-Matrix [112].

In relation to HA-based hydrogels in renal applications, the successful encapsulation of urine-derived stem cells (USCs) [83], and stem

cells intended for kidney regeneration [113] have been reported. In the latter, HA-based hydrogel microbeads were in fact fabricated with the addition of the natural polymer sodium alginate, in order to improve both the biocompatibility and degradation of encapsulated cells [113]. Gelation of the HA-alginate microbeads occurred due to crosslinking between oxygen molecules found on both sodium alginate and HA and Ca^{2+} ions found in calcium chloride (Fig. 2) [113]. Although these HA-based hydrogels were not used to ship cells long distance, but rather to induce myogenic differentiation of the cells [83], or to deliver cells to a site requiring regeneration [113], it is still worthy to note that biocompatibility of the material was reported. In fact, it can be argued that this biocompatibility can act as a starting point for future explorations into the preservation and shipment of urinary cells via the use of HA-based hydrogels.

5.4. Supramolecular hydrogels for cell encapsulation

A supramolecular hydrogel is a self-assembled structure which, unlike polymer hydrogels is formed via non-covalent interactions [114]. Typical examples of such interactions include hydrogen bonding, electrostatic, van der Waals, hydrophobic, and π–π interactions [114]. These weak reactions in turn are considered to allow for good levels of biocompatibility as well as reversible properties. This group of hydrogels can be formed using amino-acids, peptide-derived hydrogelators or proteins [115], explaining their good levels of biocompatibility. There are a number of ways in which gelation can be achieved in supramolecular hydrogels as a consequence of phase transition, including temperature, light, pH, magnetic field, electric field, redox reactions, enzymatic activity, and chemical triggers [114]. Reports have shown that the use of a supramolecular gel in cryopreservation improves viability of cells [26].

Cryopreserved MG-63 cells have previously been encapsulated within supramolecular hydrogels composed of varying concentration of protein [116]. The hydrogel basal compositions investigated were based upon genetically engineered protein-based polymers $C_2S^H{}_{48}C_2$ and $B^{RGD}C_2S^H{}_{48}C_2$ to create silk-inspired protein-based triblock copolymer hydrogels [116]. Following pH triggered neutralisation, these hydrogels formed fibrous networks which mimicked the ECM [116]. Interestingly, the level of RGD domains and adhesive motif densities incorporated within the material were both variable by design, with good levels of cellular viability reported following release after 1-, 3- and 7-days encapsulation [116]. In fact, the best levels of cell viability were found within gels containing a more concentrated level of adhesive motifs with a decreased protein concentration, resulting in gels with low levels of matrix stiffness [116]. It has previously been shown that the level of stiffness within the ECM is influential in the differentiation of stem cells [58], and more interestingly it was recently discovered that urine derived cells are capable of switching between a differentiated and undifferentiated state dependent upon their surrounding environment [117]. Therefore, when considering suitable preservative and shipment methods for UDCs, physical properties of hydrogels must be thoroughly considered and adapted where possible in order to maintain the desired biological identity of samples.

6. Conclusion and perspective

Whilst the majority of biological samples continue to be preserved and shipped under more traditional conditions (i.e. cryopreservation maintained in dry ice), investigations into alternative methods continue to offer exciting prospects for future practice. Perhaps the most intriguing ideas are those based upon the simplest of concepts. Ultimately, to be considered an effective form of sample transportation, biological integrity must be maintained throughout the entirety of the shipment process.

This starts at the very beginning of the process, meaning that cells which are intended for shipment must maintain their viability. Unlike cryopreservation, which relies on the rapid freezing of cells, this maintenance can be achieved by inducing a hibernating-like state within metabolically active cells, and has been widely demonstrated in multiple studies focused around cellular encapsulation in hydrogels. The use of hydrogels, in either ambient or sub-thermic temperatures, removes of the need for cryoprotective agents, and offers protection to the overall structure of a cell during storage and transport. Therefore, associated limitations, like the formation of ice crystals during the freezing process, can be eliminated and an improved yield of viable cells retained following shipment can be obtained. Cellular transportation in bodily fluids, as well as specialised tissue culture plastics also offer promising results, again removing the need of cryopreserved practices.

Moving forward, the opportunity to use unique devices to capture urine samples [42], transport cells in adapted multiwell dishes [41], or encapsulate cells within hydrogels as alternative preservative and shipment methods will aid in advancing the understanding of many diseases, including ciliopathies, due to improvements in sample accessibility. Recent studies have shown how beneficial the use of biomaterials can be in the preservation of biological samples, hinting at the possibility that in the near future, with increased optimisation and fine tuning, to account for any interspecies differences, cryopreservation may be a thing of the past, with hydrogels accepted as the new gold standard.

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References

[1] M.I. Maqsood, et al., Immortality of cell lines: challenges and advantages of establishment, Cell Biol. Int. 37 (2013) 10.
[2] E. Verghese, et al., Renal epithelial cells retain primary cilia during human acute renal allograft rejection injury, BMC Res. Notes 12 (2019).
[3] K.M. Ringsrud, Casts in the urine sediment, Lab. Med. 32 (2001) 191–193, 4.
[4] E. Molinari, et al., Human urine-derived renal epithelial cells provide insights into kidney-specific alternate splicing variants, Eur. J. Hum. Genet. 26 (2018) 1791–1796.
[5] S.A. Ramsbottom, et al., Targeted exon skipping of a CEP290 mutation rescues Joubert syndrome phenotypes in vitro and in a murine model, Proc. Natl. Acad. Sci. Unit. States Am. 115 (2018) 12489–12494, 49.
[6] S. Srivastava, et al., A human patient-derived cellular model of Joubert syndrome reveals ciliary defects which can be rescued with targeted therapies, Hum. Mol. Genet. 26 (2017) 4657–4667, 23.
[7] G.R. Sutherland, D. Bain, Culture of cells from the urine of newborn children, Nature 239 (1972) 199–204, 231.
[8] A. Chen, et al., Identification and characterization of two morphologically distinct stem cell subpopulations from human urine samples, Sci China Life Sci 63 (2020) 712–723. Epub ahead of print.
[9] J.Y. Choi, et al., Potency of Human Urine-derived stem cells for renal lineage differentiation, Tissue Eng. Regen. Med. 14 (2017) 775–785, 6.
[10] J. Guan, et al., Biological characteristics of human-urine-derived stem cells: potential for cell-based therapy in neurology, Tissue Eng. 20 (2014) 1794–1806, 13-14, Part A.
[11] B. Sun, et al., Therapeutic effects of human urine-derived stem cells in a rat model of cisplatin-induced acute kidney injury in vivo and in vitro, Stem Cell. Int. 2019 (2019) 8035076.
[12] S. Wu, et al., Human urine-derived stem cells seeded in a modified 3D porous small intestinal submucosa scaffold for urethral tissue engineering, Biomaterials 32 (2011) 1317–1326, 5.
[13] Y. Zhang, et al., Urine derived cells are a potential source for urological tissue reconstruction, J. Urol. 180 (2008) 2226–2233, 5.
[14] S. Rahman, et al., The FGF, TGFB and WNT axis modulate self-renewal of human SIX2+ urine derived renal progenitor cells, Sci. Rep. 10 (2020), 739.
[15] S. Tian, et al., Human urine-derived stem cells contribute to the repair of ischemic acute kidney injury in rats, Mol. Med. Rep. 16 (2017) 5541–5548.
[16] J. Guan, et al., Human urine derived stem cells in combination with β-TCP can Be applied for bone regeneration, PloS One 10 (2015), e0125253, 5.
[17] S. Bharadwaj, et al., Multipotential differentiation of human urine-derived stem cells: potential for therapeutic applications in urology, Stem Cell. 31 (2013), 9.
[18] W. Chen, et al., Skeletal myogenic differentiation of human urine-derived cells as a potential source for skeletal muscle regeneration, J. Tissue Eng. Regen. Med. 11 (2017) 334–341, 2.

[19] Q. Wan, et al., Urothelium with barrier function differentiated from human urine-derived stem cells for potential use in urinary tract reconstruction, Stem Cell Res. Ther. 9 (2018) 304.
[20] R. Lang, et al., Self-renewal and differentiation capacity of urine-derived stem cells after urine preservation for 24 hours, PloS One 8 (2013), e53980, 1.
[21] M. Pei, et al., Expansion on Matrix Deposited by Nonchondrogenic Urine Stem Cells Strengthens Repeated Passage Bone Marrow Stromal Cells' Chondrogenic Capacity vol. 356, 2014, pp. 391–403, 2.
[22] H. Steinle, et al., Reprogramming of urine-derived renal epithelial cells into iPSCs using srRNA and consecutive differentiation into beating cardiomyocytes, Am. Soc. Gene Cell Ther. 17 (2019) 907–921.
[23] T. Zhou, et al., Generation of induced pluripotent stem cells from urine, J. Am. Soc. Nephrol. 22 (2011) 1221–1228, 7.
[24] J.G. Smith, et al., Establishing acceptance criteria for cell-mediated-immunity assays using frozen peripheral blood mononuclear cells stored under optimal and suboptimal conditions, Clin. Vaccine Immunol.: CVI 14 (2007) 527–537, 5.
[25] X. Ma, et al., Slow-freezing cryopreservation of neural stem cell spheres with different diameters, Cryobiology 60 (2010) 184–191, 2.
[26] C. Zhang, et al., Hydrogel cryopreservation system: an effective method for cell storage, Int. J. Mol. Sci. 19 (2018) 3330, 11.
[27] S.P. Wheatley, D.N. Wheatley, Transporting cells over several days without dry-ice, J. Cell Sci. 132 (2019).
[28] J. Wang, et al., The analysis of viability for mammalian cells treated at different temperatures and its application in cell shipment, PloS One 12 (2017), e0176120, 4.
[29] J. Wang, et al., Transporting cells in semi-solid gel condition and at ambient temperature, PloS One 10 (2015), e0128229, 6.
[30] B. Rubinsky, Principles of low temperature cell preservation, Heart Fail. Rev. 8 (2003) 277–284, 3.
[31] Y. Petrenko, et al., Clinically relevant solution for the hypothermic storage and transportation of human multipotent mesenchymal stromal cells, Stem Cell. Int. 2019 (2019), 5909524.
[32] B.J. Fuller, et al., Biopreservation of hepatocytes: current concepts on hypothermic preservation, cryopreservation, and vitrification, Cryo Lett. 34 (2013) 432–452, 4.
[33] R. Lang, et al., Self-Renewal and differentiation capacity of urine-derived stem cells after urine preservation for 24 hours, PloS One 8 (2013), e53980, 1.
[34] P. Biberthaler, et al., The influence of organ temperature on hepatic ischemia-reperfusion injury: a systematic analysis, Transplantation 72 (2001) 1486–1490, 9.
[35] J.M. Kaths, et al., Normothermic ex vivo kidney perfusion for the preservation of kidney grafts prior to transplantation, JoVE 101 (2015), e52909.
[36] B.A. Betensky, et al., Shipment impairs lymphocyte proliferative responses to microbial antigens, Clin. Diagn. Lab. Immunol. 7 (2000) 759–763, 5.
[37] P. Prento, The effects of freezing, storage, and thawing on cell compartment integrity and ultrastructure, Hisochem. Cell Biol. 6 (1997) 543–547, 108.
[38] A. Weinberg, et al., Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization, Clin. Vaccine Immunol. 16 (2009) 1176–1186, 8.
[39] R.G. Van Buskirk, et al., Hypothermic storage and cryopreservation, Bioprocess (2004) 42–49.
[40] J. Yang, et al., The hypothermic preservation of mammalian cells with assembling extra-cellular-matrix-mimetic microparticles, J. Mater. Chem. B 5 (2017) 1535–1541.
[41] P.G. Miller, et al., A simple cell transport device keeps culture alive and functional during shipping, Biotechnol. Prog. 33 (2017) 1257–1266, 5.
[42] E. Andersson, et al., Filtration device for on-site collection, storage and shipment of cells from urine and its application to DNA-based detection of bladder cancer, PloS One 10 (2015), e0131889, 7.
[43] K. Hatherell, et al., Development of a three-dimensional, all-human in vitro model of the blood–brain barrier using mono-, co-, and tri-cultivation Transwell models, J. Neurosci. Methods 199 (2011) 223–229, 2.
[44] C. Grobstein, Morphogenetic interaction between embryonic mouse tissues separated by a membrane filter, Nature 172 (1953) 869–871, 1953.
[45] M. Birkhahn, et al., A novel precision-engineered microfiltration device for capture and characterization of bladder cancer cells in urine, Eur. J. Canc. 49 (2013) 3159–3168, 15.
[46] F.A. Coumans, et al., Filtration parameters influencing circulating tumour cell enrichment from whole blood, PloS One 8 (2013), e61774, 4.
[47] V. Bisceglie, Über die antineoplastische Immunität, Z. Krebsforsch. 40 (1934) 122–140.
[48] T. Konno, K. Ishihara, Temporal and spatially controllable cell encapsulation using a water-soluble phospholipid polymer with phenylboronic acid moiety, Biomaterials 10 (2007) 1770–1777, 28.
[49] L. Yang, et al., An agarose-gel based method for transporting cell lines, Curr. Chem. Genom. 3 (2009) 50–53.
[50] C.S. Hughes, L.M. Postovit, G.A.9 Lajoie, Matrigel: a complex protein mixture required for optimal growth of cell culture, Proteomics 10 (2010) 1886–1890.
[51] D. Hanahan, R.A.5 Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[52] K. Kim, et al., Improved isolation and culture of urine-derived stem cells (USCs) and enhanced production of immune cells from the USC-derived induced pluriopotent stem cells, J. Clin. Med. 9 (2020), 827.
[53] N. Nakamura, et al., ROCK inhibitor Y-27632 maintains the proliferation of confluent human mesenchymal stem cells, J. Peridontal Res. 49 (2013), 3.
[54] L. Zhenzhen, et al., Rho kinase inhibitor Y-27632 promotes the differentiation of human bone marrow mesenchymal stem cells into keratinocyte-like cells in xeno-free conditioned medium, Stem Cell Res. Ther. 6 (2015) 17, 1.
[55] W. Chen, et al., Microencapsulated 3-dimensional sensor for the measurement of oxygen in single isolated pancreatic islets, PloS One 7 (2012), e33070.
[56] H. Geckil, et al., Engineering hydrogels as extracellular matrix mimics, Nanomedicine 5 (2010) 469–484, 3.
[57] M.W. Tibbitt, K.S. Anseth, Hydrogels as extracellular matrix mimics for 3D cell culture, Biotechnol. Bioeng. 103 (2009) 655–663.
[58] A.J. Engler, et al., Matrix elasticity directs stem cell lineage specification, Cell 126 (2006) 677–689, 4.
[59] M. Fischer, et al., YAP-mediated mechcanotransduction in skeletal muscle, Front. Physiol. 7 (2016).
[60] K. Ren, et al., Manipulation of the adhesive behaviour of skeletal muscle cells on soft and stiff polyelectrolyte multilayers, Acta Biomater. 6 (2010) 4238–4248, 11.
[61] T.N. Clevenger, et al., Cell-mediated remodeling of biomimetic encapsulating hydrogels triggered by adipogenic differentiation of adipose stem cells, J. Tissue Eng. 7 (2016).
[62] J. Guan, et al., Biological characteristics of human-urine-derived stem cells: potential for cell-based therapy in neurology, Tissue Eng. 20 (2014) 1794–1806, 13-14.
[63] J. Zeng, et al., A supramolecular gel approach to minimize the neural cell damage during cryopreservation process, Macromol. Biosci. 16 (2016) 363–370, 3.
[64] A.I. Pravdyuk, et al., Cyropreservation of alginate encapsulated mesenchymal stromal cells, Cryobiology 66 (2013) 215–222, 3.
[65] M. Xu, et al., Secondary follicle growth and oocyte maturation by culture in alginate hydrogel following cryopreservation of the ovary or individual follicles, Biotechnol. Bioeng. 103 (2009) 378–386, 2.
[66] H. Gurruchaga, et al., Cyropreservation of microencapsulated murine mesenchymal stem cells genetically engineered to secrete erythropoietin, Int. J. Pharmacol. 485 (2015) 15–24, 1-2.
[67] J. Mumaw, et al., Rapid heterotrophic ossification with cryopreserved poly (ethylene glycol-) microencapsulated BMP2-expressing MSCs, Int. J. Biomater. 2012 (2012), 861794.
[68] H. Huang, et al., Alginate hydrogel microencapsulation inhibits devitrification and enables large-volume low-CPA cell vitrification, Adv. Funct. Mater. 25 (2016) 44, 6939-6850.
[69] E.E. Benson, et al., Alginate encapsulation to enhance biopreservation scope and success: a multidisciplinary review of current ideas and applications in cryopreservation and non-freezing storage, Cryo Lett. 39 (2018) 14–38, 1.
[70] C. de Castro Spadari, et al., Alginate nanoparticles as non-toxic delivery system for miltefosine in the treatment of candidiasis and cryptococcosis, Int. J. Nanomed. 14 (2019) 5187–5199.
[71] S. Swioklo, A. Constantinescu, C.J. Connon, Alginate-Encapsulation for the improved hypothermic Preservation of human adipose-derived stem cells, Stem Cells Transl. Med. 5 (2016) 339–349, 3.
[72] Z.M. Wu, et al., Disulfide-crosslinked chitosan hydrogel for cell viability and controlled protein release, Eur. J. Pharmaceut. Sci. 37 (2009) 198–206, 3-4.
[73] X. Yan, et al., Enzymatic Control of chitosan Gelation for Delivery of periodontal ligament cells, Macromol. Biosci. 14 (2014) 1004–1014, 7.
[74] X. Yan, et al., Periodontal tissue regeneration using enzymatically solidified chitosan hydrogels With or without cell loading, Tissue Eng. 21 (2015) 1066–1076, 5-6.
[75] Y.B. Kim, H. Lee, G.H. Kim, Strategy to achieve highly porous/biocompatible macroscale cell blocks, using a collagen/genipin-bioink and an optimal 3D printing, Process ACS Appl. Mater. Interfaces 8 (2016) 32230–33240.
[76] X. Yang, et al., Collagen-alginate as bioink for three-dimensional (3D) cell printing based cartilage tissue engineering, Mater. Sci. Eng. Part C Material Biological Applications 83 (2018) 195–201.
[77] J. Lee, et al., Development of a tannic acid cross-linking process for obtaining 3D porous cell-laden collagen structure, Int. J. Biol. Macromol. 110 (2018) 497–503.
[78] E.E. Antonie, P.P. Vlachos, M.N. Rylander, Review of collagen I hydrogels for bioengineered tissue microenvironments: characterization of mechanics, structure, and transport, Tissue Eng. 6 (2014) 683–696. Part B, Reviews, Vol. 20.
[79] A. Luca, et al., Biomacromolecular-based ionic-covalent hydrogels for cell encapsulation: the atelocollagen - oxidized polysaccharides couples, Carbohydr. Polym. 169 (2017) 366–375.
[80] Y. Ohyabu, H. Hatayama, S. Yunoki, Evaluation of gelatin hydrogel as a potential carrier for cell transportation, J. Biosci. Bioeng. 118 (2014) 112–115, 1.
[81] A. Blocki, et al., Engineering of cell-laden gelatin-based microgels for cell delivery and immobilization in regenerative therapies, Clin. Hemorheol. Microcirc. 67 (2017) 251–259, 3-4.
[82] Q. Wang, et al., Alginate/gelatin blended hydrogel fibers cross-linked by Ca2+ and oxidized starch: preparation and properties, Mater. Sci. Eng. C 99 (2019) 1469–1476.
[83] G. Liu, et al., A cocktail of growth factors released from a heparin hyaluronic-acid hydrogel promotes the myogenic potential of human urine-derived stem cells in vivo, Acta Biomater. 107 (2020) 50–64.
[84] K.H. Bae, J.J. Yoon, T.G.1 Park, Fabrication of hyaluronic acid hydrogel beads for cell encapsulation, Biotechnol. Prog. 22 (2008).
[85] L.M. Weber, et al., The effects of cell–matrix interactions on encapsulated β-cell function within hydrogels functionalized with matrix-derived adhesive peptides, Biomaterials 28 (2007) 3004–3011, 19.
[86] J. Zhu, Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering, Biomaterials 31 (2010) 4639–4656, 17.

[87] S.A. Sell, et al., The use of natural polymers in tissue engineering: a focus on electrospun extracellular matrix analogues, Polymers 2 (2010) 522–553.
[88] K. Wolf, et al., Collagen-based cell migration models in vitro and in vivo, Semin. Cell Dev. Biol. 20 (2009) 931–941, 8.
[89] M. Kirsch, et al., Gelatin-methacryloyl (GelMA) formulated with human platelet lysate supports mesenchymal stem cell proliferation and differentiation and enhances the hydrogel's mechanical properties, Bioengineering 6 (2019) 76, 3.
[90] D. Loessner, et al., Functionalization, preparation and use of cell-laden gelatin methacryloyl-based hydrogels as modular tissue culture platforms, Nat. Protoc. 11 (2016) 727–746, 4.
[91] W. Xiao, et al., Cell-laden interpenetrating network hydrogels formed from methacrylated gelatin and silk fibroin via a combination of sonication and photocrosslinking approaches, Mater. Sci. Eng. C 99 (2019) 57–67.
[92] S. Das, et al., Bioprintable, cell-laden silk fibroin–gelatin hydrogel supporting multilineage differentiation of stem cells for fabrication of three-dimensional tissue constructs, Acta Biomater. 11 (2015) 133–246.
[93] R. Schwartz, et al., Cell encapsulation in gelatin bioink impairs 3D bioprinting resolution, J. Mech. Behav. Biomed. Mater. 103 (2020) 103524.
[94] F. Ahmadi, et al., Chitosan based hydrogels: characteristics and pharmaceutical applications, Res. Pharmaceut. Sci. 10 (2015) 1–16, 1.
[95] M.C. Catoria, et al., Overview of natural hydrogels for regenerative medicine applications, J. Mater. Sci. Mater. Med. 30 (2019) 115, 10.
[96] Y. Huang, et al., In vitro characterization of chitosan-gelatin scaffolds for tissue engineering, Biomaterials 26 (2005) 36, 7616-7127.
[97] E. Assaad, M. Maire, S. Lerouge, Injectable thermosensitive chitosan hydrogels with controlled gelation kinetics and enhanced mechanical resistance, Carbohydr. Polym. 130 (2015) 87–96.
[98] M. Balyura, et al., Transplantation of bovine adrenocortical cells encapsulated in alginate, Proc. Natl. Acad. Sci. Unit. States Am. 112 (2015) 2527–2532.
[99] C. Ceccaldi, et al., Alginate scaffolds for mesenchymal stem cell cardiac therapy: influence of alginate composition, Cell Transplant. 21 (2012) 1969–1984.
[100] Y. Liu, et al., Bio-based barium alginate film: preparation, flame retardancy and thermal degration behavior, Carbohydr. Polym. (2016) 106–114.
[101] B. Chen, et al., A novel alternative to cryopreservation for the short-term storage of stem cells for use in cell therapy using alginate encapsulation, Tissue Eng. C Methods 19 (2013) 568–576, 7.
[102] A. Camboni, et al., Alginate beads as a tool to handle, cryopreserve and culture isolated human primordial/primary follicles, Cyrobiology 67 (2013) 64–69, 1.
[103] S. Jitraruch, et al., Alginate microencapsulated hepatocytes optimised for transplantation in acute liver failure, PloS One 9 (2014), e113609, 12.
[104] V. Iansante, et al., A new high throughput screening platform for cell encapsulation in alginate hydrogel shows improved hepatocyte functions by mesenchymal stromal cells Co-encapsulation, Front. Med.: Gene Cell Ther. (2018).
[105] J. Lam, N.F. Truong, T. Segura, Design of cell-matrix interactions in hyaluronic acid hydrogel scaffolds, Acta Biomater. 10 (2015) 1571–1580.
[106] S.J. Bryant, F.J.1 Vernery, Programmable hydrogels for cell encapsulation and neo-tissue growth to enable personalized tissue engineering, Adv. Healthc. Mater. 7 (2018).
[107] E.R. Horton, et al., Extracellular matrix production by mesenchymal stromal cells in hydrogels facilitates cell spreading and is inhibited by FGF-2, Adv. Healthc. Mater. 9 (2020), e1901669, 7.
[108] M.C. Schneider, et al., An in vitro and in vivo comparison of cartilage growth in chondrocyte-laden matrix metalloproteinase-sensitive poly(ethylene glycol) hydrogels with localized transforming growth factor β3, Acta Biomater. 93 (2019) 97–110.
[109] M. Kim, S. Lee, C.S.2 Ki, Cellular Behavior of RAW264.7 Cells in 3D Poly(ethylene Glycol) Hydrogel Niches vol. 5, American Chemistry Society: Biomaterials Science & Engineering, 2019, pp. 922–932.
[110] U. Blache, et al., Notch-inducing hydrogels reveal a perivascular switch of mesenchymal stem cell fate, EMBO Rep. 19 (2018), e45964, 8.
[111] F. Karimi, et al., Integrin clustering matters: a review of biomaterials functionalized with multivalent integrin-binding ligands to improve cell adhesion, migration, differentiation, angiogenesis, and biomedical device integration, Adv. Healthc. Mater. 7 (2018) 12.
[112] S. Choh, D. Cross, C. Wang, Facile synthesis and characterization of disulfide-cross-linked hyaluronic acid hydrogels for protein delivery and cell encapsulation, Biomacromolecules 12 (2011) 1126–1136, 4.
[113] J. Amirian, et al., Examination of in vitro and in vivo biocompatibility of alginate-hyaluronic acid microbeads as a promising method in cell delivery for kidney regeneration, Int. J. Biol. Macromol. 105 (2017) 143–153.
[114] J. Hoque, N. Sangaj, S.1 Varghese, Stimuli-responsive supramolecular hydrogels and their applications in regenerative medicine, Macromol. Biosci. 19 (2019), e1800259.
[115] X. Dou, C. Feng, Amino acids and peptide-based supramolecular hydrogels for three-dimensional cell culture, Adv. Mater. 29 (2017), 16.
[116] M.K. Wlodarczyk-Biegun, et al., Fibrous hydrogels for cell encapsulation: a modular and supramolecular approach, PloS One 11 (2016), e0155625, 5.
[117] O. Harari-Steinberg, et al., Ex vivo expanded 3D human kidney spheres engraft long term and repair chronic renal injury in mice, Cell Rep. 30 (2020) 852–869, 3.