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PERSPECTIVE

NANOPARTICLE-MEDIATED DELIVERY OF CRYOPROTECTANTS FOR CRYOPRESERVATION

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Abstract

Nanotechnology research has continued to garner interest and is investigated across a number of fields and industries, ranging from water treatment to clinical and biomedical applications. In biomedical research, for example, polymeric nanoparticles can be leveraged for controlled delivery of drugs and chemical compounds into cells. In cryobiological applications, polymeric nanoparticles can be utilized to deliver cryoprotectants (CPAs) and other protective agents, particularly those impermeable to the cell membrane, into cells to study their effects on cells during cooling down and warming back and at low temperatures. This perspective will discuss how polymeric nanoparticles have been used in cryobiology, with particular focus on how delivery systems have been specifically developed for low temperature applications and the potential for these systems going forward.

Keywords: nanotechnology, trehalose, CPA, cell banking, tissue banking

INTRODUCTION

Since the boom in the early 2000s, nanotechnology research has continued to garner interest and is investigated across a number of fields and industries. Nanoparticle applications range from water treatment (54), food packaging and safety (38, 58), to clinical and biomedical uses (7, 51, 55)**.** In biomedical applications, polymeric nanoparticles have been used for controlled delivery of drugs and chemical compounds into cells. For cryobiological applications, polymeric nanoparticles can be utilized to deliver cryoprotectants (CPAs) and other agents, particularly those impermeable to the cell membrane, into cells to study their effects on cells during cooling down and warming back and at low temperatures.

Trehalose, a widely investigated nontoxic alternative cryoprotectant, is impermeable to the cell membrane and mammalian cells lack the mechanism to synthesize the sugar endogenously (21, 22, 23). This small molecule is excellent cargo for nanoparticle delivery. Researchers have encapsulated trehalose using nanotechnology to carry the CPA across the cell membrane and reach the cytosol (12, 56, 75, 79, 81). Several nanoparticle formulations have been investigated, and the size, surface charge, and stimulus-responsiveness have been studied to control the cellular uptake of nanoparticles and intracellular release of their cargo.

Designing stimulus-responsive nanoparticles allows for precise release of agents, both spatially and temporally. In the past, thermally-responsive polymeric nanoparticles have typically been designed for heating/hyperthermia-associated applications. However, polymers that undergo phase changes in response to cold temperature (i.e., below ~20 °C) can be used to design nanoparticles for applications in cryobiology (66, 67). Researchers have successfully synthesized nanoparticles using the cold-responsive polymers for cell cryopreservation (12, 81).

This perspective will discuss how polymeric and other (e.g., gold and magnetic) nanoparticles have been used in cryopreservation, with particular focus on how delivery systems have been specifically developed for low temperature applications and the potential for these systems going forward.

NANOPARTICLES FOR CRYOPRESERVATION

Advances in cell-based therapies and technologies necessitate safe and nontoxic methods for both short and long-term banking of cells and tissues (6, 64). Conventional cryopreservation methods mainly rely on the use of CPAs that are able to penetrate the cell membrane, such as dimethyl sulfoxide (DMSO), ethylene glycol, propylene glycol, and glycerol. However, these CPAs have been shown to be highly toxic at body temperature and can cause adverse reactions when used for cryopreservation in cell transplantation and therapy (65, 76). Nausea, cardiac arrhythmias, neurological symptoms, and respiratory arrest have been associated with the transplantation of stem cells cryopreserved with DMSO (17). Additionally, DMSO has been found to induce differentiation of stem cells using more than 25 human stem cell lines (13). Glycerol, although less toxic than DMSO, must be thoroughly removed using a special deglycerization machine when used to cryopreserve red blood cells for transfusion (18). Rigorous and careful removal of these toxic CPAs before therapeutic use is essential. However, the washing steps add additional time and labour and may result in cell loss: up to 10% of the total number with each wash. Because of the shortcomings of these traditional CPAs, there is a great need for nontoxic CPAs that do not require thorough removal but still offer comparable cryoprotection.

One popular nontoxic alternative CPA studied is trehalose, a nonreducing disaccharide of glucose that is used by organisms to survive extreme environmental conditions (23). The ability for some small carbohydrates, like trehalose, to stabilize cell membranes and proteins during freezing and drying is attributed to two capabilities: firstly, forming hydrogen bonds with and/or promoting hydration of biomacromolecules, acting as water to allow cellular components to retain functional conformation (15, 16, 20, 39); and secondly, suspending metabolic activity by forming a glassy matrix with extremely low molecular mobility (19, 62). Trehalose has been found to offer cryoprotection to cells, but it is most effective when present both inside and outside the cell membrane (62). Unfortunately, the cellular membrane is impermeable to trehalose, and mammalian cells are not able to synthesize the sugar endogenously (73). Researchers have developed a multitude of strategies to deliver trehalose intracellularly, including engineering pores and channels (2, 8, 25, 26, 57), gene expression (29, 35), modification of trehalose itself (1), and others (60). However most approaches are non-specific, not appropriate for large quantities of cells, or may induce modifications to the cells that could alter functions (60).

Nanoparticle delivery of trehalose

Nanoparticle-mediated intracellular delivery of trehalose is an attractive method because transport across the cell membrane is specific to trehalose encapsulated in nanoparticles. Also, because it uses the cell's natural process of endocytosis, no unnecessary modification is made to the cell. A summary of studies that have utilized trehalose-laden nanoparticles to deliver trehalose into cells is given in Table 1.

Zhang et al. used a core-shell Pluronic 127-polyethylenieimine (PEI) nanocapsule to encapsulate trehalose and deliver it intracellularly to NIH 3T3 fibroblasts (79). A significant amount of trehalose was delivered into fibroblasts within 40 min of incubation at 37 °C. Thermal cycling of the cells between 37 °C and 22 °C enabled quick trehalose release, allowing intracellular trehalose concentration to reach up to 0.3 M. Cryopreservation of the trehalose-laden cells was not examined in this

Table 1. Studies using nanoparticles to encapsulate and deliver trehalose into mammalian cells**.**

Nanoparticle composition (reference)	Size (nm)	Surface charge (mV)	Cell type	Incubation time	Quality of cells cryopreserved by nTre.
PF127, PEI (79)	~100	~32	NIH 3T3 fibroblasts	40 min	N/A
PF127, chitosan, genipin (56)	~50	~17	hADSC	24 h	Comparable post-thaw viability to DMSO group. Cell stemness not different compared to fresh control.
Chitosan, TPP (75)	~250	~25	NK-92	12 h	Comparable post-thaw viability and morphology to DMSO group. Similar CD56 expression to DMSO groups. Increased cytotoxicity against leukemic cell line in NP group than DMSO group. Cell stemness not different compared to fresh control.
pNIPAM-B, PLGA, PF127 (81)	~200	-	MDA-MB-231, hADSC	4 h	Comparable post-thaw viability, 1-day viability, proliferation to conventional DMSO groups (both cell types. Cell stemness not different compared to fresh control.
pNIPAM-B, PLGA, PF127 (12)	~200	-	B-TC-6	4 h	Combined with encapsulation and magnetic rewarming. Post-thaw cell viability comparable to DMSO group. Transplanted β cells are able to regulate blood glucose levels in diabetic rat model similar to fresh control.

nTre: nanoencapsulated trehalose; PF127: Pluronic 127; PEI: polyethyleneimine; pNIPAM-B: poly(N-isopropylacrylamide-co-butyl acrylate); PLGA: poly(lactic-co-glycolic acid); TPP: sodium tripolyphosphate; hADSC: human adipose-derived stem cells

study. Although this method delivered significant amounts of trehalose in a short amount of time, nanocapsule synthesis was complex with many steps to encapsulate trehalose. Additionally, because room temperature (~22 °C) could induce trehalose release from the nanocapsules, the trehalose-laden nanoparticles are difficult to handle at room temperature for further use.

Rao et al. synthesized pH responsive genipin crosslinked Pluronic 127-chitosan (a biocompatible polysaccharide derived from chitin) nanoparticles for intracellular delivery of trehalose and successful cryopreservation of human adipose-derived stem cells (hADSCs) using trehalose as the sole cryoprotectant (56). By using genipin as crosslinking agent to crosslink chitosan, the nanoparticles could retain trehalose at room (or lower) temperature and exhibited pH responsive release at 37 °C. Cells cryopreserved using the trehalose-laden nanoparticles showed comparable post-thaw viability and stemness as those that had been cryopreserved with the traditional CPA (DMSO). However, the incubation time required for the trehalose to be released from the

nanoparticles into the cytosol for cryoprotection was lengthy – hADSCs were incubated with the trehalose-laden nanoparticles for 24 hours.

Cryopreservation of immune cells

Trehalose-laden nanoparticles have also been used to successfully cryopreserve cells designated for use in immunotherapies (75). Although natural killer (NK) cells have been cryopreserved with DMSO, special treatments are needed to maximize recovery and restore their therapeutic function post-thaw. For example, resting the cells after thawing has been reported to improve the cytotoxic functions of NK cells otherwise weakened after cryopreservation (9, 49, 52, 61), including their degranulation and killing capacity (50). Using trehalose as an alternative CPA could minimize possible CPA toxicity and adverse effects on the NK cell function. To this end, chitosan-sodium tripolyphosphate (TPP) nanoparticles were synthesized for intracellular delivery of trehalose to NK cells (75). No cytotoxicity was observed in NK cells after incubation with the nanoparticles for up to 72 h. Cell uptake studies revealed an incubation time of 12 h was necessary for cryopreservation. After cryopreservation via slow-freezing, post-thaw viability of NK cells preserved using trehalose-laden nanoparticles was comparable to that of those preserved using DMSO. Furthermore, the NK cells in the trehalose-laden nanoparticle group even possess higher capacity of target cell-killing than NK cells in the DMSO group, as demonstrated using a leukemic cancer cell line.

This study shows the feasibility of using nanoparticle-mediated delivery of nontoxic CPAs for cryopreserving immune cells and avoiding adverse effects of DMSO. However, the nanoparticle incubation time needed for sufficient cytosolic CPA concentration for cryoprotection was still lengthy, adding time to the cryopreservation procedure.

Cold-responsive nanoparticles for trehalose delivery

To shorten the incubation time necessary for sufficient intracellular accumulation of trehalose-laden nanoparticles and for the release of trehalose from the nanoparticles to the cytosol, Zhang et. al synthesized a cold-responsive polymeric nanoparticle for trehalose encapsulation, delivery, and rapid intracellular release (81). A cold-responsive polymer, poly(N-isopropylacrylamide-co-butyl acrylate), or pNIPAM-B for short, allowed disassembly of the nanoparticles upon cooling below the lower critical solution temperature (LCST, ~14-16 °C) of the polymer. The nanoparticles were synthesized by assembly of poly(lactic-co-glycolic acid) or PLGA, Pluronic F127 or PF127, and pNIPAM-B via a double-emulsion method. pNIPAM-B is insoluble above its LCST, at room temperature, which allows the pNIPAM-B, PLGA, and Pluronic F127 to form a water-in-oil-in-water structure with trehalose present in the hydrophilic/water core. However, when the nanoparticles are cooled below its LCST, the pNIPAM-B in the nanoparticle shell becomes water-soluble, triggering the disassembly of the nanoparticles and the release of encapsulated trehalose in aqueous solution.

The PLGA—pNIPAM-B—PF127 (PNP) nanoparticles were able to encapsulate a significant amount of trehalose in their hydrophilic core and release ~80% of encapsulated trehalose in response to 10 min of ice-cooling at 0 °C. Cellular uptake of the trehalose-laden PNP nanoparticles was studied in both MDA-MB-231 cells and hADSCs using cryomicroscopy. Cells were incubated with trehalose-laden PNP nanoparticles at 37 °C for various amounts of time, then moved to a temperature-controlled stage where the cells could be monitored under microscope.

As the temperature dropped below the LCST of pNIPAM-B, the PNP nanoparticles inside the cells disassembled and released trehalose into the endo/lysosome. Because trehalose is a natural osmolyte, the osmotic pressure in the endo/lysosomes should then increase, causing a net influx of water into the endo/lysosomes from the cytoplasm due to the osmotic imbalance. Consequently, the endo/lysosomes should expand, and their membranes become more permeable to allow trehalose to escape the endo/lysosomes into the cytosol. Then, the osmotic pressure in the cytoplasm should increase, leading to a net influx of water into the cell from the extracellular solution, resulting in cell volume expansion. Indeed, PNP nanoparticles-laden cells expanded as the temperature was lowered, with the extent of expansion depending on incubation time. This process was used to develop the optimal incubation time for each cell type, as too long an incubation could lead to the cell bursting if the osmotic pressure from the intracellular trehalose is too high. Optimal

incubation time for MDA-MB-231 cells and hADSCs was found to be only 4 h. It is crucial to determine the optimal incubation time for each cell type prior to cryopreservation.

To cryopreserve cells using a slow-freezing protocol, cells were incubated with trehalose-laden PNPs for 4 h, suspended in media containing 0.3 M free (i.e., non-encapsulated) trehalose. After storage in liquid nitrogen, cells were thawed and examined for quality. Immediate cell viability, 1-day cell viability, and proliferation of the cells cryopreserved using trehalose delivered using the PNP nanoparticles were all comparable to cells cryopreserved using the conventional cryoprotectant, DMSO. Although they did not outperform DMSO as a cryoprotectant, both the PNP nanoparticles themselves and trehalose-laden PNP nanoparticles did not exert cytotoxic effects at 37 °C, while DMSO is highly toxic at 37 °C. The hADSC stemness was also assessed via CD44+ and CD31- expression and multi-lineage differentiation. There was no significant difference between the stemness of cells cryopreserved with trehalose-laden PNP nanoparticles and fresh cells.

Cold-responsive nanoparticles hold great promise in the field of cryobiology. Not only does the stimulus-responsive disassembly enable spatial and temporal control over drug release, encapsulating an osmolyte such as trehalose allows for rapid endosomal escape. Additionally, using cold temperature as an external stimulus should allow for seamless integration of the cold-responsive nanoparticles into current cryopreservation protocols. Because of the relatively quick uptake and rapid disassembly in response to cold, the PNP nanoparticles do not add exorbitant time or labour to the slow-freezing cryopreservation process. Rather, they achieve cryopreservation results comparable to DMSO without the need for cell washing in order to eliminate the potentially harmful penetrating CPA. PNP nanoparticles may also have the potential to be incorporated into vitrification or rapid-freezing protocols, but further studies are needed.

There is also potential to modify the LCST of the cold-responsive polymer and therefore, change the temperature below which the PNP nanoparticles would dissemble and release the encapsulated agent. This would allow for the study of nanoparticle uptake and cargo release at different temperatures.

Enhancing cryopreservation with nanoparticle

Nanoparticle-mediated delivery of trehalose can be used in combination with other cryopreservation techniques, like hydrogel encapsulation and magnetically heating, to enhance outcomes and better facilitate cell-based

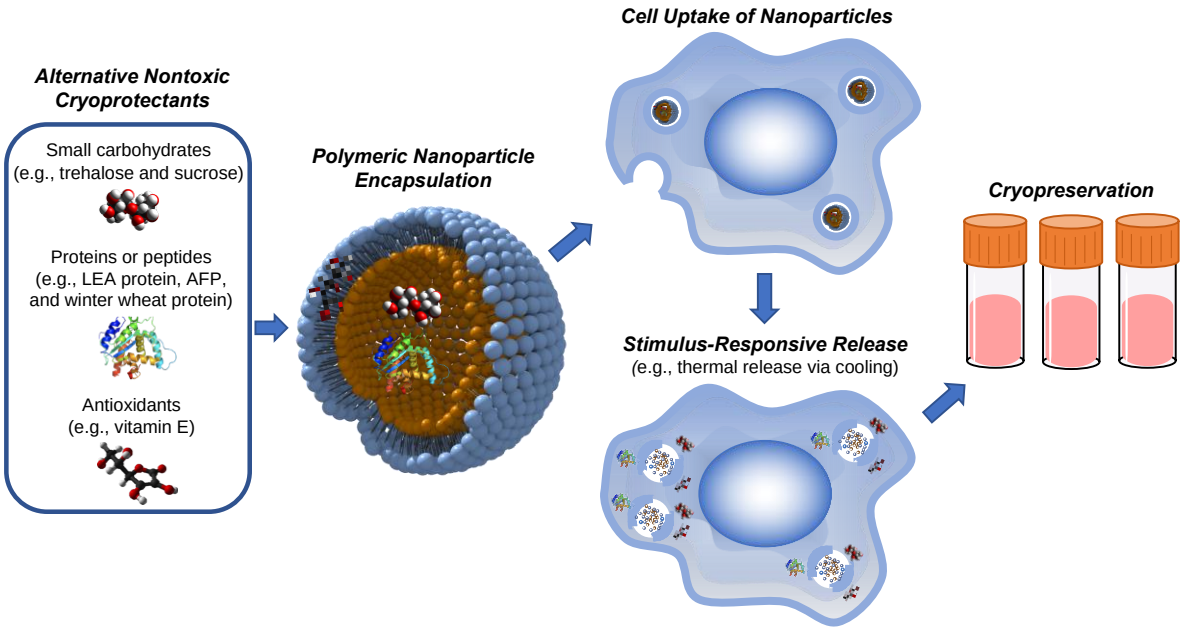

Figure 1. Schematic illustration of the use of polymeric nanoparticles for cell cryopreservation. LEA: Late embryogenesis abundant; AFP: Antifreeze protein.

therapies.

Encapsulating cells in a 3D hydrogel network has been initially used to protect cells against mechanical stress and possible immune rejection during cell transplantation (53, 59). More recently, it was found that some hydrogels (e.g., calcium alginate) can minimize physical damage to cells by restricting growth of ice crystals and inhibiting ice recrystallization during cooling and rewarming (40, 80), which has been utilized to enhance cell cryopreservation (10, 40, 47, 71, 74, 78, 80, 82). Researchers have developed efficient methods for cell encapsulation in hydrogels via electrospray and droplet microfluidics, with mild gelation conditions that do not impact cell viability (40, 41, 44, 80, 82).

Enhancing the rewarming process during cryopreservation using magnetically inducted heating (MIH) or laser-induced heating can also prove beneficial (10, 42, 47, 48, 70). Magnetic, gold, or other nanoparticles can be distributed through the sample for uniform heating of the frozen sample at the nanoscale under external stimulation (e.g., magnetic field and laser) which not only improves the warming rate but also may inhibit ice recrystallization to reduce cryoinjury during cryopreservation (47). In addition, some nanoparticles (e.g., graphene oxide) have been shown to absorb onto ice crystals to restrict the growth and recrystallization of ice crystals (30).

Cheng et al. synthesized a cold-responsive, pNIPAM-B-based nanocapsule (CR-NC), in combination with alginate hydrogel encapsulation and MIH, to use trehalose as the sole cryoprotectant for successful cryopreservation of β cells (12). A 4-h incubation with the trehalose-laden CR-NCs was sufficient for β cells to accumulate enough intracellular trehalose to offer cryoprotection. Cells containing trehalose-laden CR-NCs were then encapsulated into an alginate hydrogel and cryopreserved via slow freezing. Magnetically responsive iron oxide (Fe_3O_4) nanoparticles were incorporated into the extracellular trehalose solution surrounding the cell-laden hydrogels, to enhance the rewarming process. The combination of trehalose-laden capsules, hydrogel encapsulation, and MIH enabled high post-thaw cell viability, adherence, and proliferation. Cryopreserved β cells showed normal insulin production similar to fresh cells as evidenced by immunofluorescence. The quality of cryopreserved cell-laden hydrogels was further confirmed by transplantation therapy in diabetic rats. Cryopreserved cell-laden hydrogels were able to regulate blood glucose levels for roughly two weeks, similar to fresh cell-laden hydrogels. This study, together with others (10, 47) demonstrates the versatility of nanoparticle-mediated trehalose delivery for cell cryopreservation and its great potential when combined with other established techniques for augmenting cryopreservation.

OUTLOOK AND CONCLUSIONS

The nanoplatforms developed for trehalose-mediated cryopreservation detailed here are exciting and promising. However, only a handful of studies have explored the capability of nanoparticles-mediated CPA delivery for cell cryopreservation (Table 1). The potential of nanoparticles for cell preservation merits further exploration. Figure 1 conceptualizes the procedure of using polymeric nanoparticles for cell cryopreservation. First, nanoparticle delivery of trehalose for its use as the sole cryoprotectant has only been demonstrated in a limited number of cell types (< 5), so more research is needed to test the capability of the approach for cryopreserving a number of different types of cells. Second, trehalose holds great potential for preserving cells not only via cryopreservation, but lyopreservation, since trehalose is a main mechanism used by organisms that can survive desiccation (21, 22, 23).

Banking cells at ambient temperature with the aid of nanoparticle-trehalose delivery would be a monumental achievement in cryobiology and ground-breaking for facilitating cell-based medicine and therapy. Because cell preservation at ambient temperature is more challenging than cryopreservation, trehalose alone may not confer sufficient lyoprotection to cells. Anhydrobiotes have been shown to use a variety of protective proteins in addition to trehalose to survive extreme conditions (5). Nanoparticle co-delivery of trehalose and late embryogenesis abundant (LEA) proteins, both used in organisms able to withstand desiccation (4, 24, 28, 43, 72), may be needed (11, 31, 37, 46).

Trehalose and LEA proteins are not the only alternative CPAs that merit exploration for nanoparticle-mediated delivery. Depending on the synthesis and composition, nanoparticles can be designed to encapsulate hydrophilic agents, hydrophobic agents, or both simultaneously (68,

69). Encapsulation of hydrophilic proteins in the core of polymeric nanoparticles could protect the proteins from premature degradation or denaturation (77). Co-encapsulation of proteins with trehalose, a natural osmolyte, could allow for protein endo/lysososomal escape (81), to avoid protein degradation in the endo/lysosomes. Co-delivery of multiple agents may allow for improved post-thaw cell viability and/or quality through combination of agents with different protective mechanisms. Antioxidant supplementation of freezing medium has been shown to increase the quality and viability of cells after cryopreservation (3, 27, 63). Antioxidants may confer protection to cells against oxidative stress during freezing. Intracellular nanoparticle-delivery of antioxidants may prove especially beneficial to transplanted cells, as they can improve cellular functionality that may be impaired during cryopreservation (45). Additionally, proteins derived from winter wheats, wheat varieties that are more tolerant to extreme freezing conditions than spring varieties, have been shown to improve cryopreservation outcomes in several studies (14, 32, 33, 34, 36). Nanoparticle-mediated delivery of these agents would allow for further study of the effects these compounds separately or in combination on cell preservation.

In conclusion, the application of nanotechnology for the study and advancement of cryopreservation shows great promise. Cold-responsive nanoparticles have the potential for easy integration into cryobiology applications, since cooling to cold temperature is naturally done during cryopreservation. In combination with other modern technologies such as hydrogel encapsulation, laser/magnetically induced heating, and carbon nanomaterials, the cold responsive nanoparticle-mediated delivery of nontoxic CPA and other protective agents may have tremendous potential to advance the field of cell/tissue/organ banking by not only cryopreservation but also lyopreservation.

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