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Apatite nanoparticles strongly improve red blood cell cryopreservation by mediating trehalose delivery via enhanced membrane permeation

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ABSTRACT

Cryopreservation of red blood cells (RBC) is an important method for maintaining an inventory of rare RBC units and managing special transfusion circumstances. Currently, in a clinical setting, glycerol is used as cryoprotectant against freezing damage. After thawing and before transfusion, glycerol must however be removed to avoid intravascular hemolysis, via a complex and time-consuming deglycerolization process which requires specialized equipment. Improved cryopreservation methods using non-toxic agents are required to increase biocompatibility and decrease processing time. Biocompatible cryoprotectants (e.g. trehalose) were proposed, but their low permeation through RBC membranes limits their cryoprotection efficacy. Herein, we report for the first time a glycerol-free cryopreservation approach, using colloidal bioinspired apatite nanoparticles (NP) as bioactive promoters of RBC cryopreservation mediated by trehalose. Addition of apatite NP in the medium tremendously increases RBC cryosurvival, up to 91% (42% improvement compared to a control without NP) which is comparable to FDA-approved cryoprotection protocol employing glycerol. NP concentration and incubation conditions strongly modulate the NP bioactivity. Complementary experimental and computational analyses of the interaction between apatite NP and model lipid bilayers revealed complex events occurring at the NP-bilayer interface. Apatite NP do not cross the bilayer but momentarily modulate its physical status. These changes affect the membrane behavior, and promote the permeation of trehalose and a model fluorescent molecule (FITC). This approach is a new alternative to using toxic glycerol for cells cryopreservation, and the identification of this enhancing no-pore permeation mechanism of apatite NP appears as an original delivery pathway for cryoprotectant agents and beyond.

1. Introduction

Transfusion of red blood cells (RBC) is essential for patients necessitating blood supplies [1], in particular after traumas or surgical procedures, but also in cases of blood-related pathologies such as anemia or leukemia [2]. Viable RBC supplies must be ensured by blood banks, requiring the setup of cell preservation methodologies [3,4]. Low-temperature approaches allow reaching high rates of cell preservation [5,6], up to 85–90%; however, current approved RBC cryopreservation methods require high concentrations of glycerol as cryoprotective agent (e.g. 40% w/v in

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North America) [2]. Glycerol has been studied as cryoprotectant for RBC cryopreservation for decades and its efficacy as cryoprotectant, the mechanism of its protecting ability, as well as its effects on cells, tissues and patients as a whole have been well documented [6–8]. However, because of the toxicity of glycerol, which can generate side effects such as hemolysis or RBC shape alterations [9], the use of cryopreserved RBC for transfusion necessitates a complex and lengthy procedure of deglycerolization prior to the use in patients [10]. This deglycerolization process requires multiple washing steps where about 15% cells are hemolyzed [6]. Also, published studies pointed out the adverse changes in RBC morphology after cryopreservation and deglycerolization [10,11]. Moreover, glycerol residues remain inside the cells despite this post-treatment (<1% for North America and <0.8% for Europe) [6], which can provoke complications in some patients. These considerations prevent the widespread development of cryoprotective approaches for providing large quantities of RBC to hospital units in minimal timescales [4]. Nowadays such approaches are limited to rare blood types.

Thus, improved cryopreservation methods based on non-toxic cryoprotective additives which would not require long post-processing are needed to optimize RBC preservation and allow better availability of blood to patients.

Alternative biocompatible cryoprotectants including biopolymers such as polyvinylalcohol (PVA) [12] or sugars [13–15] like trehalose have been proposed, but their cryoprotective abilities remain limited [12] for practical relevance and approval from regulatory commissions (expected survival typically > 80%) [2]. Trehalose is a particularly appealing bio-inspired cryoprotectant as it is well known for its role in protecting cells and organisms against a variety of stresses, including freezing, desiccation, heat shock, oxidative and osmotic stresses [16,17]. However, in order to provide maximum protection, trehalose must be present on both sides of the cell membrane; yet it has very low permeability to RBC membrane under normal conditions [16]. Therefore, research has focused on methods for delivering trehalose into cells, i.e. via the use of trehalose-loaded liposomes [15], membrane-permeable biopolymers [13], electro-permeabilization [18], and chemical methods [16], but research is still in progress for attaining high cell recovery after freeze-thawing.

So far, there have been no reports on the use of inorganic-based compounds for the delivery of trehalose into cells. Yet, bioactive and biocompatible inorganics have already been found particularly useful in various biomedical domains (e.g. nanomedicine, medical imaging/theranostic, regenerative medicine, etc.) [19,20]. Possible inorganic compounds for delivery of trehalose to RBC could be calcium phosphates (CaP), which are known for their excellent biocompatibility, biodegradability and bioactivity [21–24]. Moreover, it was demonstrated that biomimetic (bone-like) apatite nanoparticles (NP), which possess structural and chemical similarity with the mineral component of bones and dentin [25], are particularly efficient carriers of drugs and nucleic acids into various types of cells, making them a promising candidate for further investigations as nanocarriers of payloads in cells [22,26–29]. The proposed mechanism of apatite NP assisted delivery of bioactive molecules in cells is generally based on endocytosis-mediated transport. However, endocytosis does not exist in mature mammal RBC [30], meaning that delivery of trehalose into RBC assisted by apatite nanoparticles should occur via a different mechanism, for example via a local modification of RBC membrane physical properties. The nano-size and charge distribution of apatite particles may modulate their interaction with RBC cells [31], although this was reported so far only for the specific case of heparin-coated hydroxyapatite, in another scientific context, and in the absence of cryoprotectants. To-date the possibility to use apatite NP for enhancing RBC survival has not been investigated.

Previous work has shown that apatite nanoparticles could be formulated as colloidal suspensions via the formation of an external organic corona surrounding the particles. 2-aminoethylphosphate (AEP) as well as phosphonated polyethyleneglycol have been shown to be efficient stabilizing agents to form individualized colloidal apatite NP [23,28]. In the case of AEP-stabilized apatite NP, positively-charged particles are obtained due to the presence of an amino group on the AEP molecule; however the addition of hexametaphosphate (HMP) anions was found to improve significantly the dispersibility of the particles, facilitate their purification (e.g. via dialysis process) and favor colloidal stability [22,32]. This effect can be explained by the organization of HMP anions around the AEP-stabilized particles, thus leading to the formation of a double-layer (positive/negative) organic corona on the surface of apatite NP as illustrated in Fig. 1a.

Because of this peculiar distribution of positive and negative charges around the particles, AEP/HMP-stabilized apatite NP might lead to specific interactions with cell membranes, possibly modulating momentarily the properties of the membrane, which could in turn promote the permeation of trehalose (see general concept on Fig. 1b). Based on this hypothesis, and taking into account good hemocompatibility of colloidal apatite NP [33], the aim of the present work was to investigate, for the first time, the possibility of using AEP/HMP-stabilized colloidal apatite nanoparticles for facilitating the delivery of trehalose into RBC, in view of promoting their cryopreservation without the use of glycerol. This paper reports experimental data on the role of colloidal apatite NP on RBC viability after freeze-thawing cycle and explores, at the molecular scale and by using synthetic lipid bilayers, the mechanism of interactions between RBC and apatite NP. These experiments were completed by the use of computational methods, with the aim to explain our experimental observations. The set of complementary data is presented and discussed, to demonstrate the active role of colloidal apatite NP for RBC cryopreservation.

2. Results

2.1. Characterization of the colloidal apatite nanoparticles used in this work

After preparation, the AEP/HMP-stabilized colloidal nanoparticles (NP) prepared (see Materials and Methods section) were characterized from a physico-chemical viewpoint using complementary techniques. DLS measurements indicated that the mean NP size followed a monomodal distribution centered on d_{50} ~ 40 nm (Fig. 2a), with d_{10} ~ 22 nm and d_{90} ~ 80 nm.

Zeta potential measurements systematically showed an overall negative surface potential of the order of −50 to −55 mV. This can be explained by the exposure of HMP anions organized on the organic corona at the periphery of the particles, and covering the positively charged AEP adsorbed molecules (Fig. 1a) [22]. XRD analyses pointed out the apatitic nature of the particles by comparison to reference data [25,34], and no additional crystalline compound was detected indicating that the samples are single-phased (Fig. 3a). FTIR results corroborated XRD conclusions by the presence of characteristic phosphate vibration modes related to calcium phosphate apatite (Fig. 3b) [35]. The clear detection (Fig. 3c) of HPO_4^{2-} vibrational contributions in the region 530-550 cm^{-1} (as well as at ~ 875 cm^{-1}), as in bone mineral [25], along with the limited contribution of OH^- ions at 632 cm^{-1} are indicative of the nonstoichiometric character of the apatite phase [25], pointing to the bio-inspired nature of these particles. A low degree of carbonation (arising from atmospheric CO_2) may also be detected in the region 1400-1600 cm^{-1}, corresponding to ~ 1 wt. % carbonate