

HES - A Nightmare?

Getting older from year to year, I sometimes wake up at night because of the following dream: I am sitting in my office preparing one of these terribly urgent reports. Suddenly the telephone rings.

Question: May I ask you some questions?

Answer: Yes, for sure (grimacing). What's up?

Question: What is hydroxyethyl starch (HES)?

Answer: HES is a modified natural polymer of branched amylopectin (one of the two components of starch, the other component is the linear amylose). Its physical and chemical characteristics are mainly defined by i) the degree of hydroxyethylation (DS), i. e. replacement of hydroxyl groups of the anhydroglucose units by hydroxyethyl groups) and ii) the molecular weight distribution (MW). Whereas native starch is hardly water soluble, hydroxyethylation increases water solubility. DS is determined by measuring the number of substituted anhydroglucose units and dividing this number by the total number of anhydroglucose units in the molecule. The molar substitution (MS) is calculated by measuring the total number of hydroxyethyl groups present and dividing this by the total number of anhydroglucose units. DS and MS are not the same, but they are often incorrectly used interchangeably in the literature. As hydroxyethylation can occur at carbon positions 2, 3 or 6 of the anhydroglucose unit, depending of the manufacturing process, the substitution pattern can vary greatly. HES molecules show a great polydispersity (in contrast to other cryoprotectants such as glycerol or dimethyl sulfoxide (Me_2SO), which have molecular weights of 92.09 g/mol and 78.13 g/mol, respectively). The molecule sizes of HES usually follow a sort of bell-shaped distribution, ranging from some thousand g/mol to over a million g/mol. Consequently "the molecular weight" can be regarded as an "average" molecular weight only. There are two ways for the calculation: i) arithmetic mean, i. e. total weight of all molecules divided by the number of molecules (M_n) and weight averaged mean (M_w). Again, both of them are often incorrectly used interchangeably in the literature.

Question: So what's hiding behind an HES specified as "450/0.7"?

Answer: It should be an HES with an M_w of 450,000 g/mol and an MS of 0.7 (i.e. 70 hydroxyethyl groups per 100 anhydroglucose units of the polymer). A comprehensive overview can be found in Banks et al. (The structure of hydroxyethyl starch. Br. J. Pharmacol. 47: 172-178 (1973). Suitable for a long rainy day.

Question: Who was the first one to use HES for the cryopreservation of biological cells?

Answer: To the best of my knowledge: Knorpp et al. (Hydroxyethyl starch: Extracellular cryoprotective agent for erythrocytes, Science 157: 1312-1313 (1967). They compared the efficacy of HES to that of polyvinyl pyrrolidone (PVP), another polymer which had been used for volume replacement in humans but grew out of fashion because of its hepatotoxicity. Previously Rinfret and coworkers (e.g. Factors affecting the erythrocyte during rapid freezing and thawing, Ann. N. Y. Acad. Sci. 85: 576 (1960) had conducted extensive studies on boiling heat transfer with a cryogenic fluid that would preserve red cells suspended in various extracellular additives including the above-mentioned PVP.

Question: Which types of cells have been frozen so far using HES?

Answer: Various mammalian cell lines (including human haematopoietic cell lines, keratinocytes, Chinese hamster ova cells (CHO), spermatozoa and bone marrow derived as well as peripheral blood stem cells, mononuclear cells (lymphocytes and monocytes), platelets, and red cells. Some people have even claimed to be able to successfully freeze human granulocytes using a combination of HES and Me₂SO (e.g., Lionetti et al., Factors affecting the stability of cryogenically preserved granulocytes. *Cryobiology* 17: 297-310 (1980): However, no clinical application has resulted so far. Nowadays the only routine clinical application of HES as a cryoprotectant is for the freezing of blood stem cells at slow cooling rates in combination with 5% Me₂SO.

Question: Is there a good source for HES and are there any to be avoided?

Answer: Never use a product named "Hydroxyethyl starch", product code H 6382 in the catalogue 2000/2001 from SIGMA. Only an average of 10 per 100 anhydroglucose units are substituted with a hydroxyethyl group (DS = 0.1). As a consequence it is poorly soluble in water. You may use a product which is called "Hetastarch", product code H 2648, from the same company. It comes as a 6% solution in 0.9% sodium chloride. However, this is incredibly expensive (approx. EUR 15 per gram HES) compared to what you have to pay when you ask people in the intensive care unit of your local hospital: They have used HES for many decades as a volume replacement after blood loss (and then the costs are approx. EUR 0.33 per gram HES). The modifications familiar for this purpose are 450/0.7, 200/0.5 and 70/0.5. The concentrations vary from 3 to 10% (wt/v) and the electrolyte content is adjusted to isotonicity (mostly by adding sodium chloride). However, if you want to work with the pure dry substance, you have to dialyse it.

Question: Why that?

Answer: The solutions available for volume replacement contain low molecular weight impurities (LMWI, i.e., electrolytes, oligosaccharides). LMWI differ not only from one HES modification to the other, sometimes they even differ from one lot to the next from the same manufacturer. This is one reason why some people have difficulties to reproduce the work of others (and sometimes their own...).

Question: I have no idea how to do the dialysis procedure. Has it been published somewhere?

Answer: Yes. We have published this - unfortunately in a German journal (*Z. Klin. Med.* 46, 1567-1570 (1991)). But an English translation of the dialysis procedure is available upon request. In principle you may use dialysis tubes from any supplier, as long as the cut-off is about 10,000-14,000 g/mol (e.g., from SIGMA, product code 250-7U or 250-9U). We found tubing closures (e.g. from SIGMA, product code Z37,095-9) to be helpful, too. Sometimes it is really a problem to knot the ends, and the tubes are subject to cracking especially after the procedure. Make sure that you fill in HES solutions at concentrations not higher than 10% (wt/wt), otherwise half of the tubes will crack. Wash the tubes carefully prior to use. Never use the tubes for more than one dialysis procedure. And wear a lab-coat when doing the procedure with large volumes for the first time!

Question: This sounds rather cumbersome. Couldn't you provide me with some dialysed dry substance?

Answer: Sorry. We have no dry substance at the moment ourselves. But I could send you some 250 ml of a 25% (w/v) HES solution (= 57 g) for free. It's KryoHAES, optimised for

the cryopreservation of red cells. We used it in our last clinical trial (Horn et al. Transfusion of autologous, hydroxyethyl starch-cryopreserved red blood cells. *Anesth. Analg.* 85: 739-745 (1997)). There are some bottles left. The high HES content (23% w/w) and the low electrolyte content (60 mmol/l) allow the investigation of the influence of sodium chloride concentrations above 60 mmol/l without problems. But if you want lower NaCl concentrations and/or HES concentrations higher than 23%, you have to perform the above mentioned dialysis procedure.

Question: Whereas there is some knowledge regarding the action of penetrating cryoprotectants such as glycerol and Me₂SO, what is the mechanism of this macromolecular compound which does not lower the freezing point?

Answer: The two most important (at least from my point of view) are: First of all, in the presence of HES a certain amount of water does not crystallize. As a consequence, the solute enrichment in the extracellular space (which takes place due to the phase transition of water into ice during the freezing process = hypertonic stress) is lowered. For the above mentioned HES the amount of water which does not crystallize is roughly 0.5 g per gram HES (Koerber *et al.* The influence of hydroxyethyl starch on ice formation in aqueous solutions. *Cryobiology* 19: 478-492, (1982)). Secondly, there may be an interaction between the cell membrane and HES, resulting in a stabilizing effect. HES is a highly polar compound due to its numerous hydroxyl groups. These hydroxyl groups form hydrogen bonds with the polar compounds of the cell membrane, a stabilizing effect especially if the concentration of "free" (= liquid) water is reduced.

Question: Is it a problem to determine cell viability after thawing in the presence of HES?

Answer: Yes.

Question: What does HES make so interesting that you have worked on it for over 17 years now?

Answer: Well, at first I was fascinated about the unconventional chemistry and behaviour of this substance. But from a clinical point of view, it is biocompatible so it does not have to be removed after thawing. All techniques developed so far for the freezing of red cells require a time-consuming process for deglycerolization prior to the transfusion. My dream is...

This is usually the point when I wake up. And it happened this time, too. So I decided to write down this nightmare to get rid of it once and for all!

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