Improvements in Polysaccharides for use as Blood Plasma Expanders

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Summary: Modern blood plasma volume expanders consist of water-soluble polysaccharides that are compatible with the human body. They are more effective for the treatment of intravasal volume deficiency compared to synthetic polymers. These colloidal blood plasma volume expanders, for example pullulan, dextran or hydroxyethyl starch (HES) are used in blood isotonic electrolyte solutions. HES has the lowest tendency to remain in the liver or other organs of the human body in comparison to other expanders.
The knowledge of the molar mass and coil dimensions as well as their distribution are essential, since products of low molar masses do not have the desired effect and parts of large molar masses can lead to an anaphylactic shock. Injection of HES enhances the microcirculation and leads to a better oxygen transport into the tissue and the muscle. Because of this, HES was identified as a doping agent in 02/2002. In addition to this it will be shown that starch acetates have an even better physiological compatibility and are an interesting alternative to HES. We will present investigations on the shelf life of a new acetyl starch, which may be an alternative to the blood plasma expanders used to date.

Keywords: acetyl starch; blood plasma volume expander; hydroxyethyl starch; molar mass distribution; particle size distribution

I. Introduction

To ensure circulation functions normally within the bloodstream, a sufficient filling volume of the vascular system has to be guaranteed. A decrease in the amount of blood circulating caused by any kind of polytrauma leads to a decreasing internal pressure in the vascular system and causes a decreasing venous back-current that may result in a state of shock or at worst even death.[1] Blood loss does not necessarily need to be compensated for by blood transfusion.[2] This treatment is only essential if the blood loss exceeds 30 % of the total blood volume and/or if the hemoglobin concentration drops below 100 g/l (normal concentration is 140 to 160 g/l).[3] Treatment with whole blood is still the best method to increase the blood volume because colloids and electrolytes, as well as immunoglobin and blood clotting agents are supplied in the right proportions. However, whole blood can be supplied in finite amounts only. Problems that can arise with blood transfusions include incompatibility of the different blood types as well as the risk of infections (for example HIV or hepatitis). In addition to this, major technical effort is required to ensure secure acquisition (testing on phatogens), storage and transport.[4] For the treatment of the volume deficiency of the vascular system, crystalline (for example sodium chloride) or artificial colloidal plasma expanders are used. The advantages of these two groups of plasma enhancing agents compared to whole blood are their almost infinite availability, their extended shelf life, their lack of dependence on blood type and their

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absence of phatogens. Whether crystalline or colloidal blood plasma expanders are more useful has been the subject of intense discussion in literature in recent years.[5–7]

The development of erythrocyte-free infusion solutions started with the observation that death on blood loss is not caused by the loss of red blood cells but by the loss of blood volume itself.[8] In the beginning (in 1881), solutions of common salt were used as blood plasma expanders. In the same decade it became evident that common salt solutions led to serious swelling of the stomach. These problems and the knowledge that the colloidal osmotic pressure of the proteins is of utmost importance led to an intensive search for a suitable colloid as a plasma expander agent. With the outbreak of World War I in 1914, the first colloid-based blood plasma expanders on the basis of arabic gum solutions (Bayliss 1915/Hurwitz 1915) and gelatine (Hogan 1915) were developed. However, due to biological contamination, these products were not able to compete on the market. Human albumin was also used but because of the danger of infections (hepatitis, rabies, HIV) and its low availability comparable with whole blood, its importance has decreased over the years. Today we are aware of another problem with gelatine. Recently it was reported that Bovine Spongiform Encephalopathy (BSE) may be caused by the prions crossing the intestinal wall into the body by coupling at the Fe-atom of the ferritin.[9] If this is proven, then we can expect gelatine to disappear from the market in the near future.

With the outbreak of World War II, new products like polyvinyl pyrrolidone (PVP) entered the market. However, this synthetic polymer could not be metabolized in the human body and was therefore deposited in the tissue and organs. It was tested on 500,000 patients until the 1980's without any monitoring of the physiological consequences. Unfortunately, it is still used in Taiwan.[10]

This contribution shows the suitability of different polysaccharides for the application as blood plasma expanders. In the next section the functionality of polymeric blood plasma expanders is demonstrated.

II. Functionality of a Polymeric Blood Plasma Expander

As already described in the introduction, a blood plasma expander operates via the colloidal osmotic pressure π induced by the macromolecules. This is shown schematically in Fig. 1.

If the colloidal osmotic pressure π_1 in the blood vessel is as high as the osmotic pressure π_2 in the surrounding tissue the *volume effect* is 100%. If the osmotic pressure in the blood vessel is higher than that in the tissue, tissue water migrates into the blood vessel and the volume effect increases beyond 100 % (see Table 1).

It is important to note that the standard human organism (5–6 l blood amount) can

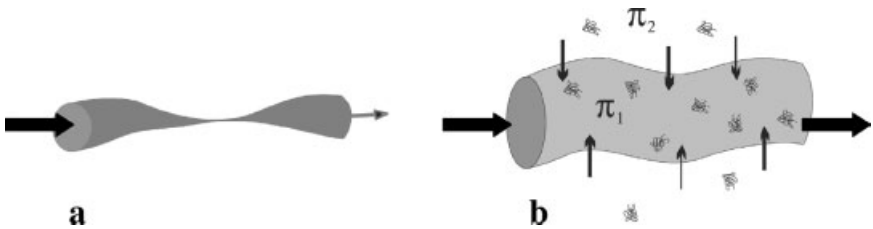


Figure 1.
Functionality of a blood plasma expander. **a**: After a polytrauma the blood vessel collapses because of pronounced blood loss. The blood circulation is disturbed dramatically; **b**: Polymers enhance the colloidal osmotic pressure in the blood vessel, water migrates into the blood vessel from the surrounding tissue and the blood circulation is no longer disrupted.

Table 1.
Specifications of commercially available HES samples [29].

HES	70/0.5	130/0.4	200/0.5	200/0.5	200/0.62	450/0.7
Concentration	6%	6%	6%	10%	6%	6%
Volume effect	70%	100–130%	100%	130%	100%	100%
Effective period (h)	1–2 (short)	3–4 (middle)	3–4 (middle)	3–4 (middle)	5–6 (long)	5–6 (long)
Molar mass (g/mol)	70,000	130,000	200,000	200,000	200,000	450,000
DS	0.5	0.4	0.5	0.5	0.62	0.7
C2/C6 relationship	4:1	9:1	6:1	6:1	9:1	4,6:1

deal with a blood loss of less than 0.5 l by itself. Up to this amount, common salt solutions can be used to balance the loss of blood. However, if polytrauma occurs due to a severe accident or other circumstances, the immediate blood loss or the blood loss caused by lengthy surgical operations is far higher so that a blood plasma volume expander has to be used in order to maintain the blood circulation. When the blood loss exceeds about 30 % of the total blood volume (approx. 2 l), whole blood has to be used instead of blood plasma expanders because the proteins and ions have to be substituted as well.[1]

Before blood plasma enhancing agents can be used, it is essential to determine the absolute molar mass and its distribution together with the coil size and its distribution. A polymer that is to be used as a blood plasma expander should not have a weight-average molar mass M_w below 40,000 g/mol because it would then be excreted by the body via the kidneys. Blood plasma enhancers with low molar masses around 40,000 g/mol are only used for tinitus [11]. However, the greatest problem with the molar mass is the high molar mass fraction of a polymer.[12] It has been shown in the past, that the high molar mass fraction leads to anaphylactic reactions of the organism which result in a pronounced risk to life.[13] The polysaccharides used have also to be optimized in terms of their solubility. Only ''perfect'' water-soluble samples can be used as blood plasma expanders. In the case of starch derivatives, it is important to remember that the solubility depends on the degree of substitution (DS). A small DS (<0.4) may lead to partial insolubility, which is life-threatening because it may result in vascular obliteration. If the DS is very high (>0.8), the samples prepared are no longer metabolized, which would result in an unwanted accumulation in the human body.

III. Experimental Section

The intrinsic viscosities $[\eta]$ were determined with an Ubbelohde capillary Ic in a water tub conditioned at $25.0 \pm 0.05\,^{\circ}C$. A knowledge of the intrinsic viscosities is of utmost importance in order to determine the appropriate Kuhn-Mark-Houwink-Sakurada relationship ($[\eta]$-M-relationship) [14]. Another advantage of viscosimetric measurements is that viscosimetry proves to be the right tool to easily determine the shelf life of a polymer via single point measurements [15] of the coil dimensions, as can be seen in Fig. 9.

Determination of the molar mass and its distribution was performed using hyphenated assemblies [16] of size exclusion chromatography (SEC, 4 columns TSK PWXL: G3000, G4000, G5000 & G6000, ToSoHass), multi-angle laser light scattering (MALLS, DAWN-F light scattering photometer, Wyatt Technology) and differential refractometry (DRI, Showa Denko), as shown in Fig. 2. The absolute determination of the molar mass becomes necessary since a relative determination via SEC is not sufficient as there are no water-soluble standard systems for the starch derivatives investigated in this work. The picture of the light scattering device was taken from.[17]

In the apparatus, the samples are first degassed before passing through a damper and a 0.02 μm filter and entering the autosampler. They then pass through a

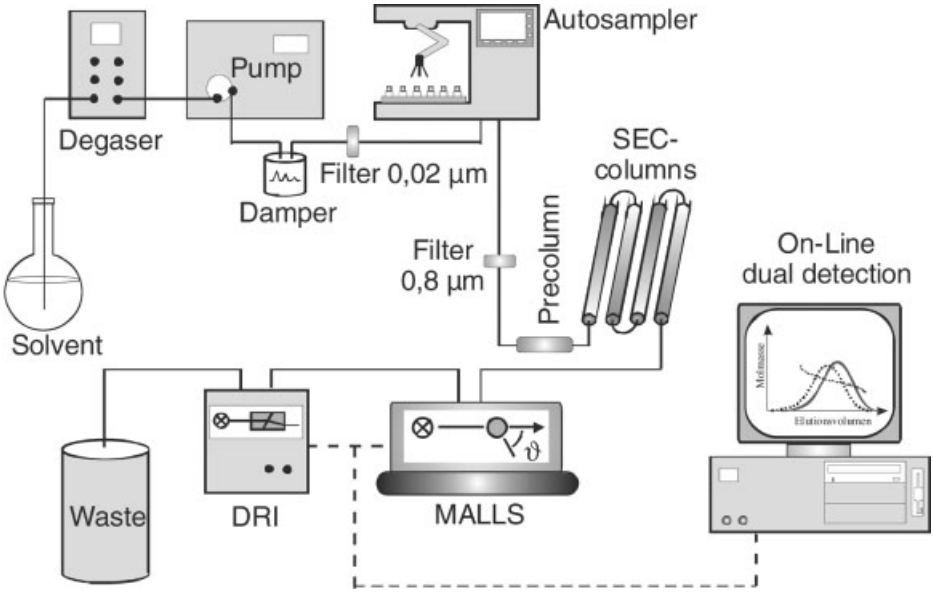


Figure 2.
Hyphenated Techniques [16,17] of SEC/MALLS/DRI for the determination of the molar mass and the coil size, as well as their distributions.

0.8 µm filter and a precolumn before entering the SEC columns, where they are separated into a large number of molar mass fractions. The fractions are then analysed in terms of molar mass and particle size in a multi-angle laser light scattering apparatus combined with a differential refractometer. The results are evaluated on a PC and the absolute distributions of the dual detection are displayed on screen.

Fig. 3 shows a comparison of the separation efficiency of three different column sets for the polymer standard pullulan in water. As can be seen here, the column set TSK-Gel shows the best separattion of the standard pullulan into its molar mass slices, since the dependence of the molar mass from the elution volume shows the largest linear regime. Although the column set from Suprema also exhibits a linear regime, the slope for the TSK-Gel columns is smaller, resulting in a better separation. The column set PL-aquagel-OH did not prove to be the right means of separation for the water-soluble standard pullulan. Consequently, the column set TSK-Gel was used for the characterization of the polysaccharides investigated in this work, for details see[16] and[18].

IV. Results and Discussion

As mentioned in the outset, whole blood is of course the best substitute because it consist of colloids, electrolytes and coagulation factors in the correct proportions. However, there are numerous problems: the availability of different blood stocks is limited, there is a risk of infection, blood is expensive and the shelf life is restricted to about six weeks. Replenishment with blood is necessary if the volume lost is greater than approx. 2 litres. On the other hand, isotonic sodium chloride solutions are used if the blood loss is less than about 0.5 litres, and this situation occurs every day in hospitals. If blood loss exceeds 0.5 litres, polymers can be used, which have the advantage that they generate colloid osmotic pressure. This causes water from the tissue to fill the blood vessels. This effect has been known for a considerable time and as a result hydrocolloids were used, such as Arabic gum and polylvinyl pyrrolidone.

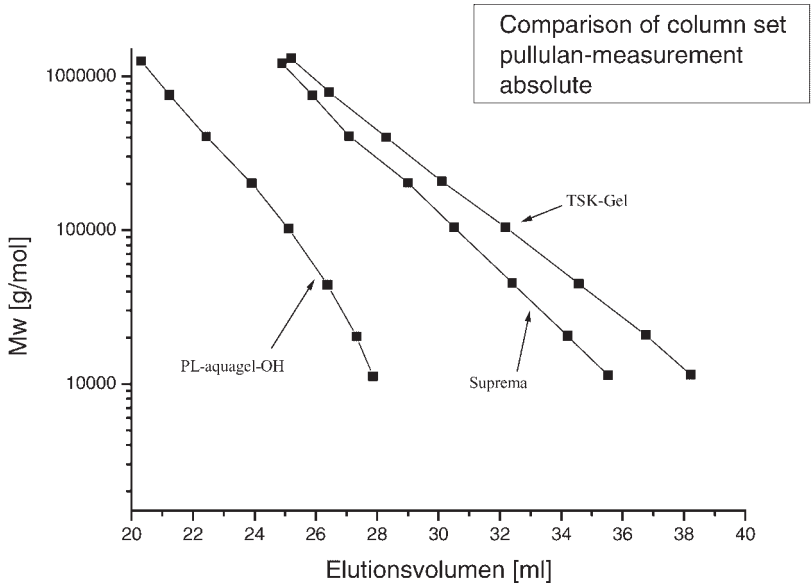


Figure 3.
Calibration of the column sets for the hyphenated technique of SEC/MALLS/DRI with pullulan standards.

They have now been superseded because the danger of infection in the case of human albumin cannot be disregarded. Gelatine, which is still on the market, is also the focus of suspicion, as reported in December last year.[9] At present, polysaccharides are favoured because of their good properties and may be used as blood plasma expanders.

In the last few decades, **dextran** has been one of the most frequently used blood plasma expanders. It has been shown that using dextran has resulted in anaphylactic reactions.[19,20] These are life-threatening and are caused by the high molar mass fractions ($M_w > 200{,}000$ g/mol). To keep the volume effect and microcirculation at an optimum, the molar mass and the concentration for a perfectly soluble dextran sample have to be adjusted. This holds true for any polysaccharide which is to be used and is still the subject of detailed and intense investigations. An important therapeutic effect of the intravenously administered polysaccharides is their specific interaction with low-density lipoproteins by means of weak van der Waals forces, which results in the elimination of the lipid fraction from the blood compartment.

There have also been several attempts to develop blood plasma substitutes based on **pullulan**.[21] Shingel[22] summarized all efforts which have been made so far to understand the pharmacokinetics of intravenously applied pullulan in terms of the molar mass and concentration. Pullulan has the disadvantage, compared with other polysaccharides, that the viscosity yield exceeds that of blood by far (molar mass > 150,000 g/mol led to a rapid increase in venous pressure) and that a reduction in the molar mass leads to a rapid exclusion from the organism (for molar mass < 15,000 g/mol the half-time from injection to urinal excretion is very short, resulting in a higher effort for the kidneys), followed by the development of secondary hemorrhagic shock. Therefore, the refined polymer should be free from low and high molar mass components. More recently, it has been shown that pullulan exhibits a great affinity toward the liver and is effectively endocytosed by the parenchymal liver cells.[22,23] Hepatic uptake of intravenously administered pullulan is markedly reduced by the co-administration of asialofetuin and arabiogalactan having a high affinity to the

corresponding cell receptors, meaning that pullulan, in contrast to dextran, penetrates the hepatocyte membrane via receptor-mediated endocytosis.

One example of an unaltered polysaccharide that could be used in the future is **ι-carrageenan**.[24] Since this biopolymer has not passed the medical tests yet, the emphasis still lies on starch derivatives. In the past it has been shown that **ethyl starch** (ES) was not suitable for use as a blood plasma expander because of its insufficient solubility in water.[25]

Hydroxyethyl Starch (HES)

At present, the polymer that is used most as a blood plasma expander is hydroxyethyl starch[26] **HES**, market share in Germany ≅80 % in 2004). This starch derivative has surpassed dextran in terms of market share in recent years. Whereas dextran had a market share of 44 % in 1986 it fell to 2 % in 1996.[27] HES as an ether derivative is used in a wide range of molar masses from around 15,000–500,000 g/mol and a degree of substitution (DS) ranging from 0.4–0.7 (see for example Table 1 for details, together with volume effects and C2/C6 ratios). A DS of 0.4 is the minimum value for HES to exhibit good dissolving statistics. Above a DS of 0.9 HES is no longer perfectly soluble and becomes toxic for the organism as it tends to accumulate and is therefore stored in essential organs.[28] The volume effect exhibits a maximum of 130 % and the effective period is 5–6 h at maximum. The C2/C6 ratio shows a maximum value of 9:1 for the samples 130/0.4 and 200/0.62. The ratio is very important because C2 limits attack by amylase. The C2 position exhibits strong steric hindrance whereas the C6 position shows only weak steric hindrance, see Fig. 4. Therefore the stability during application is often given by this ratio, see Table 1.

Although **HES** is a very sufficient blood plasma expanding agent and also exhibited the fewest side effects compared with the others, it was shown that because of the very solid ether bond not all of the polymer is degraded by the amylase and excreted via the kidneys, but that tissue storage was observed not only for rats [30] but also for dogs and pigs [4]. Pictures of these tissue deposits are shown together with those appearing in the lung and kidneys in Fig. 5. For human otological patients HES accumulations were also found in the tissue [31].

Summing up the properties of HES, one can conclude, on the basis of the molecular parameters, that it seems to be an appropriate blood plasma expander, but that more than a few disadvantages occur. These disadvantages include anaphylactic reactions, as well as accumulation in the tissue (resulting from highly substituted fractions,

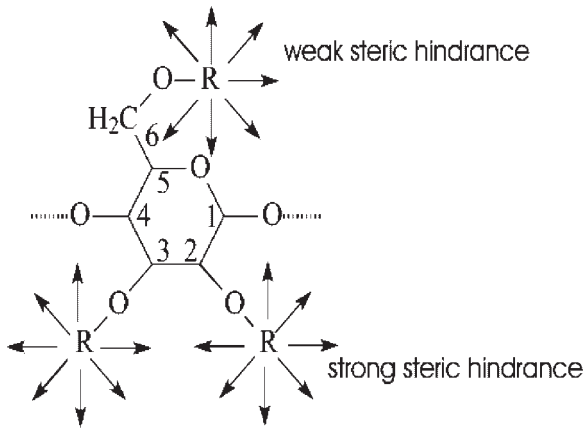


Figure 4.
Steric hindrance for substituents on the anhydroglucose unit (AGU) of hydroxyethyl starch.

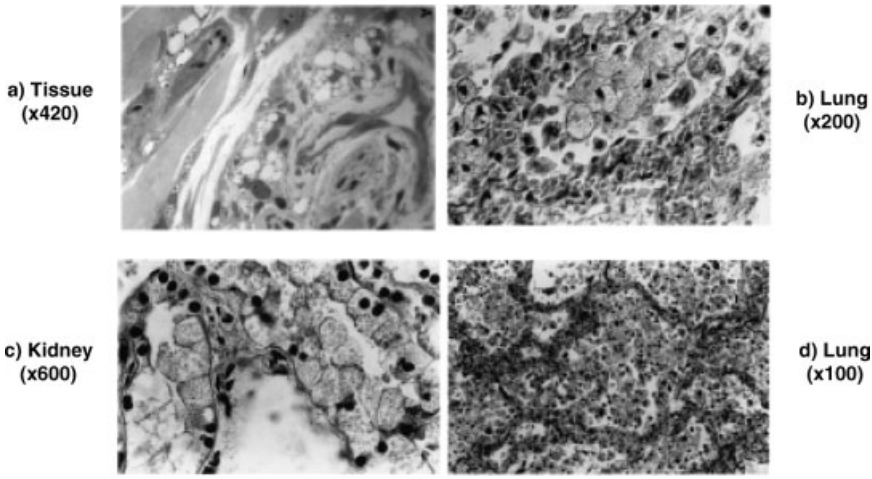


Figure 5.
Deposits of HES in tissue, kidney and lung, pictures taken from pig tissue, published in [32] and [33].

as the substitution pattern of the degraded polymer is not homogenous along the chain) leading to severe therapy-resistant itching and deposits in the very important reticuloendothelial system.

Reiman et al.[37] recently reported itching that lasted for several months and was resistant to therapy after HES had been administered. The reason for this is that the substitution of hydroxyethyl starch is not homogeneous along the chain so that after the degradation of the backbone, HES fragments result with an approximately 10% higher degree of substitution and these are deposited in the tissue. For this reason, attempts have been made to use other substances, namely acetyl starch.

Acetyl Starch (AS)

A relatively new starch derivative that is to be used as blood plasma expanding agent is acetyl starch (**AS**, for the molecular structure see Fig. 6). **Acetyl starch** has already been synthesized in the past with $DS_{C2} \neq DS_{C3} \neq DS_{C6}$.[34] In this case $C_3 > C_2 \gg C_6$! It has been tested and shown that it is effective as a blood plasma expander but that it cannot be stored over a lengthy period of time.[4]

Due to the ester-bond of the acetyl group compared to the ether-bond of the hydroxyethyl group in **HES**, the advantage of **AS** should be a better degradability with a constantly good plasma enhancing property.

The reason is that no enzymes exist in the human body that are capable of splitting the ether bond. In contrast to this, the ester bonds can be hydrolysed by esterases. Therefore in the case of AS storage in the tissue or other organs is no longer a problem.[35] One problem with **AS** is the inferior shelf life of the polymer[35] wen substitution takes place at C2, C3 and C6. As there is only minor steric hindrance for C6 (see Fig 4 for details), the attack of the amylase will take place at this position.

As tested in an excellent research work by Warnken et al.[36] and shown in Fig. 7, the normally substituted AS is suitable for use as blood plasma expander, as it can be readily metabolized by the human organism. After a period of time of about 24 h the concentration in the organism is far below a critical value. On the other hand, Fig. 7 shows that these AS samples are not stable in terms of long-lasting storage, as acetic acid is separated in large amounts at standard temperatures of 20 °C and 40 °C. The aim for AS should therefore be to synthesize a sample that is only substituted at position C_2, assuming that the shelf life

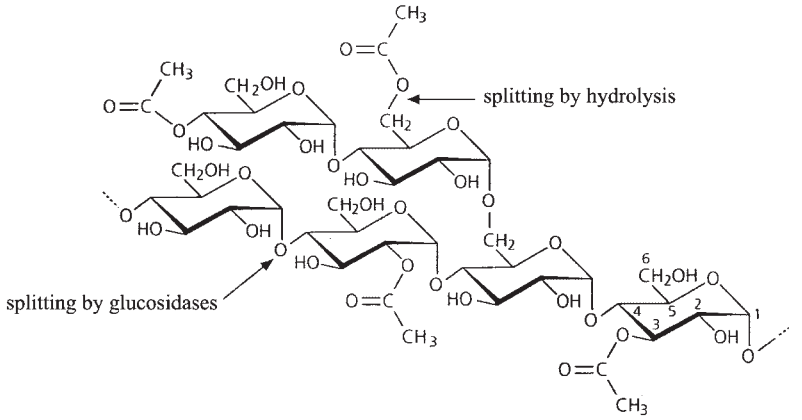


Figure 6.
Molecular structure of the blood plasma expander acetyl starch (AS).

will be increased dramatically so that storage will no longer be a problem.

2-O-Acetyl Starch (AS)

The synthesis of 2-O-acetyl starch cannot be discussed in detail here because our patent application is still pending. After we had successfully synthesised about 80 samples, we were able to show that all these samples were synthesised in the correct regiospecific position at C2.

Evidence for the selectivity of the reaction was gained from NMR spectroscopy, both by means of ^{1}H NMR of the acetyl starch as well as ^{1}H-broadband decoupled ^{13}C NMR spectroscopy of the acetyl starch sample in DMSO-d6, as shown in Figure 8. In the ^{13}C NMR spectrum of a 2-O-acetyl starch with a degree of substitution of 0.93, only signals for the pure acetyl starch are found and no impurities could be detected. The CH_3 moiety of the acetate yields a signal at 21 ppm. The carbonyl carbon of the ester function shows a peak at 171 ppm. All other signals are caused by the repeating unit carbons (C-1 to C-6). No hints for substructures from side reaction or any impurities were found.

The selectivity can be concluded from the occurrence of only one carbonyl peak for an acetate moiety in C-2 at 171 ppm. A splitting pattern would be observed in the

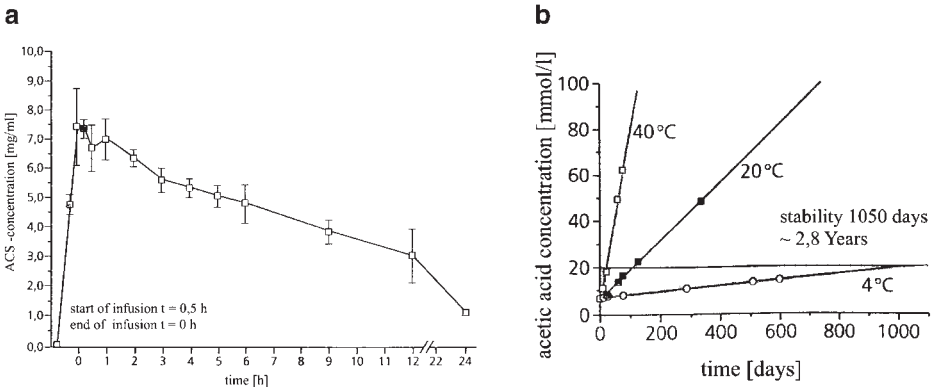


Figure 7.
a: AS-concentration versus time after infusion of 500 ml AS 200/0.7, 6 % w/w on 4 volunteers, determination of concentration via SEC, HPLC and RI detection. b: Time-dependent determination of acetic acid in stored, sterilized AS-solutions. (Figure taken from Reference No. 36).

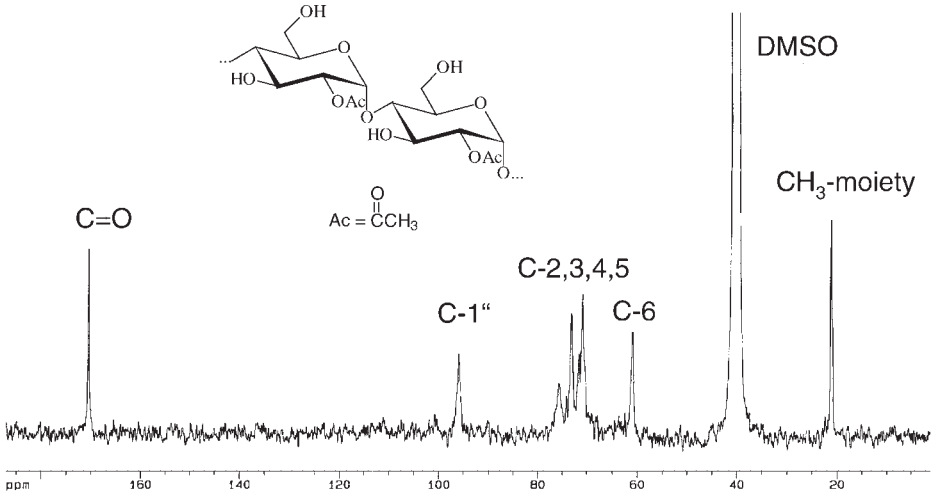


Figure 8.
^{1}H-broadband decoupled ^{13}C-NMR spectrum of a 2-O-acetyl starch. DS = 0.93. The ^{13}C spectrum was acquired with a Bruker Avance 400 spectrometer using inverse-gated (composite pulse) proton decoupling.

case of additional acetylation at C-3 and C-6. Moreover, only one signal for an unmodified C-6 unit is determined at 60.9 ppm. The C-1 signal is shifted to higher field (lower ppm values) because of the functionalization of the adjacent OH- group in C-2. Thus, it occurs at 96 ppm in contrast to a C-1 having no acetyl group in the neighbourhood which would give a signal at about 98–100 ppm.

It should also be mentioned that the purity of the acetyl starches was investigated using Fourier-Transform infrared spectroscopy (FTIR) (DMSO-d_6 5000 scans).

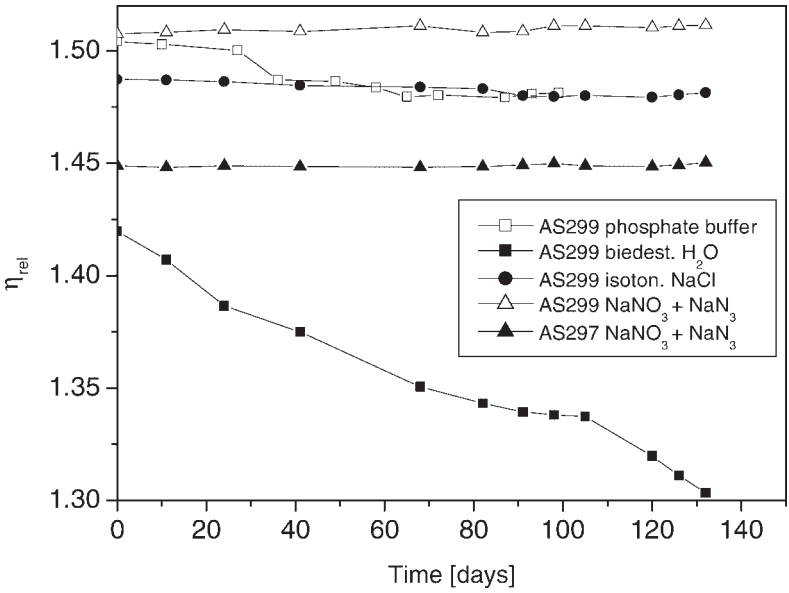
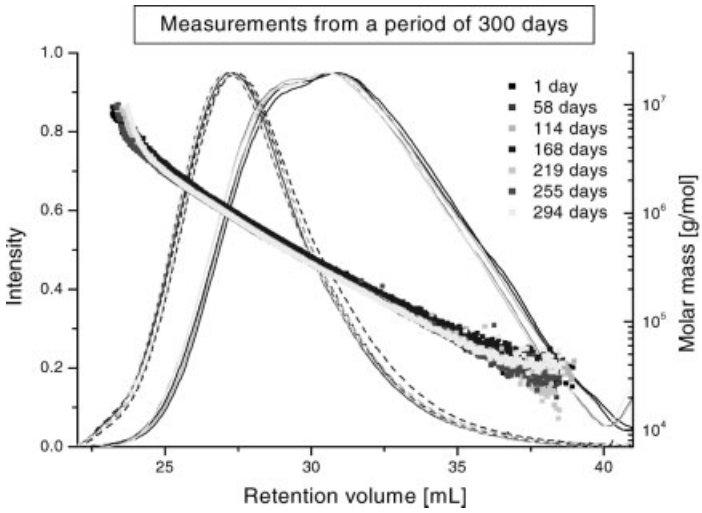


Figure 9.
Determination of the storage stability of acetyl starch (AS) via viscosimetric single-point measurements of the relative viscosity.



period [days]	M_w [g/mol]
1	426.000
58	422.000
114	415.000
168	411.000
219	405.000
255	403.000
294	396.000

Figure 10.
Evolution of the molar mass with time (300 d) for the blood plasma expander acetyl starch (AS) investigated with hyphenated techniques of SEC/MALLS/DRI. (C = 0.022 g/mL for all samples).

Apart from the typical cellulose signals and the carbonyl signal of the acetate at 1725 cm^{-1}, the method was also used to specifically examine the purity of the acetates. No impurities were found in any of the sample, which indicated that the samples produced in this way are free of contamination.

Storage Stability

Since the 2-O-acetyl starch was made available, the question was if this starch derivative shows the desired storage stability over a long period of time. Fig. 9 shows viscosimetric single point measurements of the relative viscosity for two different acetyl starches in various solvents. Except for the

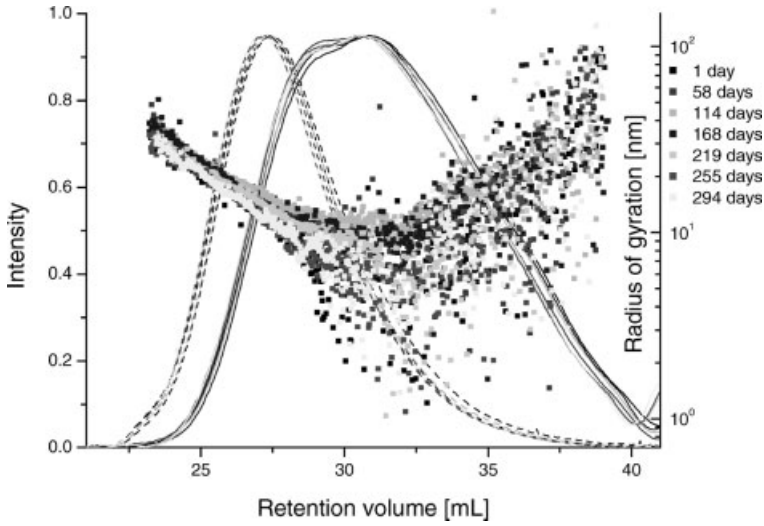


Figure 11.
Evolution of the radius of gyration with time (300 d) for the blood plasma expander acetyl starch (AS) investigated with hyphenated techniques of SEC/MALLS/DRI.

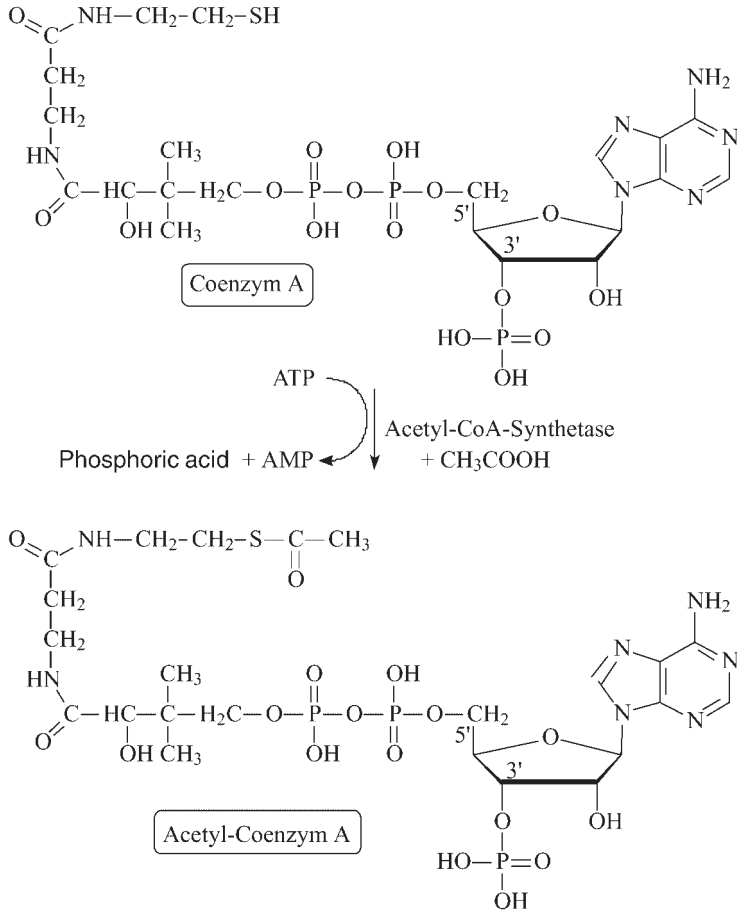


Figure 12.
Mechanism of the enzymatic determination of the amount of acetic acid that is separated from the acetyl starch (AS).

sample AS 299 in water, which was contaminated by amylase, all samples show a constant relative viscosity η_{rel} over a period of time of 140 days, meaning that the solution conformation of the polymer coil does not change within the time frame investigated. This result reflects the storage stability of the respective **AS** samples.

In Fig. 10 the storage stability of one investigated **AS** is tracked via light scattering measurements with hyphenated techniques of SEC/MALLS/DRI over a period of 294 days. The broken lines in Figures 10 and 11 show the signal from the differential refractometer, and the solid lines the laser light scattering signal at 90°.

As one can see in Fig. 10, the weight-average molar mass M_w of the investigated **AS** stays almost constant within the error margins of the method. The molar mass decreases only very slightly from 426,000 g/mol to 396,000 g/mol within the investigated time frame of 294 days.[25]

The left-hand side shows the intensity and the right-hand side the molar mass versus retention volume. No significant change was observed, particularly in the high and low molar mass tails.

The same trend, shown in Fig. 10 for the molar mass, is shown in Fig. 11 for the coil size. As one can see from this picture, the coil size keeps a constant value within the

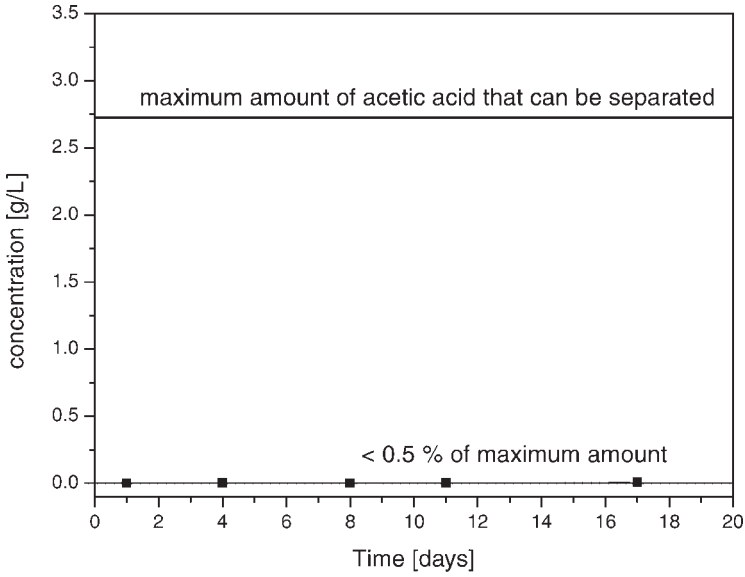


Figure 13.
Enzymatic determination of the amount of acetic acid that is separated from the acetyl starch (AS) over a time period of 17 days (C = 0.02 g/mL). The commercially available kit from Boehringer Mannheim (Order No. 0148 261) was used for the determination.

investigated time frame of 294 days.[25] The scattering data for the radius of gyration versus retention volume above 25 mL show that measurements are being made at the sensitivity limits of the laser. From 20–28 mL there is no significant change in the coil size and this agrees with the viscosity measurements (see Fig. 9).

Another very suitable method to quantitatively determine the storage stability of acetyl starch is to measure the amount of acetic acid that is separated from the polymer via hydrolysis of the relatively unstable ester bond. As shown in Fig. 12, this process can be monitored via enzymatic methods incorporating the adenosine triphosphate (ATP) catalysed transition from coenzyme A (CoA) and acetic acid to acetyl-coenzyme A (active acetate). The amount of acetic acid can then be monitored indirectly via UV/VIS spectroscopy, as the extinction of the nicotinamide-adenine dinucleotide (NAD) lies at 340 nm.

This method was used to determine the amount of acetic acid that is separated from one distinctive acetyl starch over a time period of 17 days. The results are shown in Fig. 13 and document the good storage stability of the investigated AS at least for this period of time, as the amount of acetic acid determined this way accounts for less than 0.5% of the maximum amount of acetic acid that can be separated from the polymer.

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