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CH30043: Advanced Practical Chemistry

The Latest Natural and Synthetic Polymeric Advances in Protamine Replacements for Heparin Neutralisation after Cardiopulmonary Bypass Surgeries.

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ABSTRACT

Heparin is a naturally occurring anionic polysaccharide which has uses as an anticoagulant drug in cardiopulmonary bypass surgeries. Protamine Sulphate, a shellfish protein, is the only registered antidote. The arise of adverse effects after protamine usage, including anaphylactic shock and hypotension has led to the search for a new heparin antidote. Due to its largely anionic charge and high polydispersity, heparin has proved a hard target to synthesise a new drug for which is both effective and safe, and new developments often lead to largely cationic drug molecules that are highly cytotoxic *in vivo*. This review will focus on the latest developments of protamine replacements; specifically, naturally derived and synthetic polymers, and the advantages and disadvantages of these.

Supervisor: Dr. Stephen Bromfield

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Glossary

a. **Zymogen** - A substance which is inactive until activated by an enzyme. The newly activated substance then acts as an enzyme itself.

b. **Antithrombin (AT III)** - A protease that inhibits coagulation by neutralising factors involved in the coagulation cascade, including thrombin.

c. **Blood Complement** - Circulating blood proteins involved in the immune system.

d. **Biocompatibility** - The effect of interaction of biomolecules in the human body.

e. ***In vitro* testing** - Tests done on biomolecules outside of their normal environment, i.e., in glassware.

f. ***In vivo* testing** - Tests carried out inside of a living organism.

g. **aPTT** - 'Activated partial thromboplastin time'; an *in vitro* test that determines the amount of time taken for blood clots to form by use of blood plasma. The amount of time taken can be affected, for example increased by adding anticoagulants (heparin), or decreased by adding coagulants (protamine).

h. **Fluorescein labelling -** Attachment of a fluorescent molecule to the drug candidate before administration of the drug *in vivo*. Excitation of a portion of blood at the specified wavelength leads to an amount of fluorescence, which will be proportional to the concentration of the drug in that sample.

i. **Cytotoxicity** - The toxicity of a substance towards cells.

j. **Anti Xa assay** - A measure of the concentration of heparin in the blood. This test is preferred for heparin sensing in surgeries over the aPPT test which can underestimate heparin concentrations in the blood.

k. **LD_{50}** - 'Lethal dose'; the dosage of a drug that kills 50% of the test subjects.

l. **Host-guest complex** - An aggregate of a small molecule (guest) bound to a complex (host) through non-covalent interactions.

m. **Indicator displacement assay** - A test to measure binding affinity of a molecule to a target. An indicator is complexed to the target molecule. The molecule being tested is added to the complex. The change in absorption due to generation of the free indicator molecule on binding of the test molecule to the target is measured by UV-Vis to determine the binding ability of the molecule.

n. **Dynamic light scattering (DLS)** - A technique used to determine the size of particles.

o. **Atomic force Microscopy (AFM)** - A test used to form a 3D image of particles in high resolution.

p. **Isothermal titration calorimetry (ITC)** - Determines the binding ability of a molecule to a macromolecule by measuring the temperature change on reaction of the substances; an increase in temperature indicates bonds being formed; so the larger the temperature increase, the greater the binding affinity.

q. **Fibrinogen** - A protein that is converted to fibrin; the main structural feature of a blood clot.

r. **Block copolymer** - A polymer made up of alternating sections of two or more polymers made of different monomer units.

s. **MTT assay** - A colorimetric test used to measure the viability of cells by measuring their ability to undergo metabolic processes through reduction of a dye, leading to a change of colour.

t. **Fibroblast cells** - A structural cell involved in wound healing.

u. **CMC** - The 'critical micelle concentration'; the concentration of a surfactant molecule required for the molecules to aggregate and form a micelle.

Introduction

Unfractionated Heparin (UFH), a naturally occurring negatively charged polysaccharide, is an anticoagulant drug administered during cardiopulmonary bypass surgeries[1] (clinical dose of approx. 300 IU/Kg) to inhibit blood clotting. At the end of surgeries, heparin needs to be removed from the body in order for the coagulation cascade to restart to prevent excess bleeding in the patient. Alternative heparin anticoagulants used for scenarios other than surgery include low molecular weight heparin (LMWH), and Fondaprinux. Heparin acts on the coagulation cascade (Figure 1), a series of zymogen[a] activations leading to the formation of a blood clot.[2]

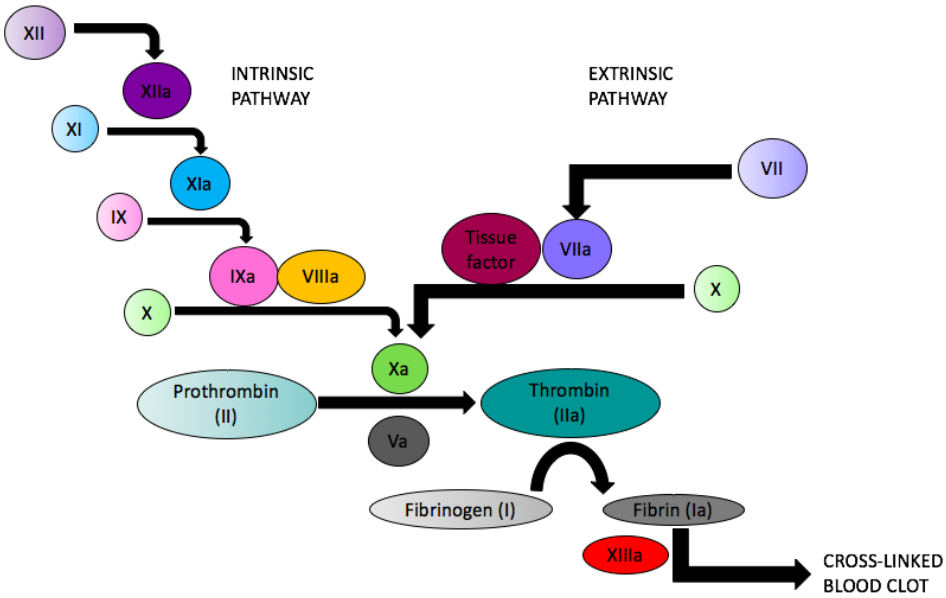

Figure 1. The factors involved in the coagulation cascade.

A pentasaccharide sequence in heparin (figure 2) activates antithrombin III[b] (ATIII) by binding to a positively charged pocket and thus causing a conformational change on ATIII. An allosteric site on ATIII now has a high affinity for binding to many activated enzyme factors, including XIIa, XIa, IXa, Xa, and thrombin, all of which are involved in the coagulation cascade. The mechanism of action is thought to occur through the formation of ternary heparin-ATIII-thrombin complexes.[2-6] The irreversible binding of ATIII to these factors inhibits the formation of thrombin, a key molecule in formation of a thrombus, resulting in the inhibition of clotting.

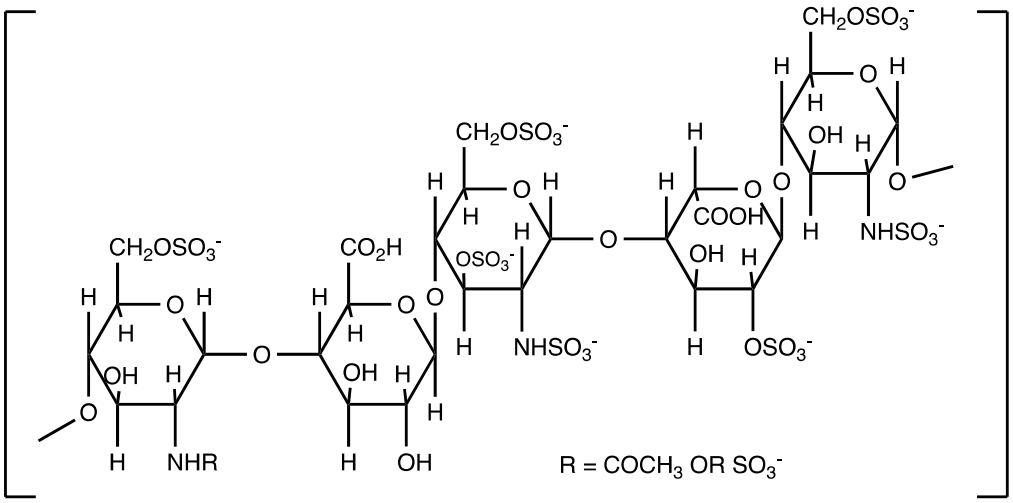

Figure 2. The pentasaccharide unit responsible for binding to Antithrombin III leading to activation of the allosteric site on AT.[3]

Currently, the only clinically approved antidote for heparin neutralisation in cardiopulmonary bypass surgeries is a naturally occurring largely cationic protein extracted from shellfish, protamine sulphate, more simply known as protamine.[7] Protamine however, is shown to have many adverse effects. These include hypotension; immune responses including antibody production and anaphylactic shock, caused by large heparin-protamine complexes activating blood complement[c]; anticoagulation properties at higher concentrations of protamine, and weakened blot clot formation.[8-11] Therefore, the search for an alternative to protamine that is both safe, and effective, is currently of great interest for researchers.

It is not only protamine that causes complications however. The structure of heparin itself (Figure 3) is undefined as it has a high polydispersity, and therefore the exact composition and amount of the drug administered is unknown. This is further complicated by the binding of anionic heparin to blood components such as proteins and white blood cells, instead of acting on the coagulation cascade.[12,13] The heparin chains are made up of sulphated uronic acid and glucosamine disaccharide units, and the number of negatively charged sulphate groups per chain varies, making heparin a hard target to develop a new drug for.[13] In addition, administration of heparin is associated with a great risk of bleeding.[14]

Whilst the new antidote drug must have a highly cationic nature if it neutralises heparin through the binding of many electrostatic interactions, researchers are less certain on the structure type that would be most effective in the neutralisation of heparin. Previously, many approaches have been taken in attempts to find a solution, for example small drug-like molecules such as the pentacationic peptide delparantag, however this was discontinued after it failed phase II clinical trials when it was found to cause hypotension[15], deeming its safety no longer superior to that of protamine's which exhibits the same side effect.

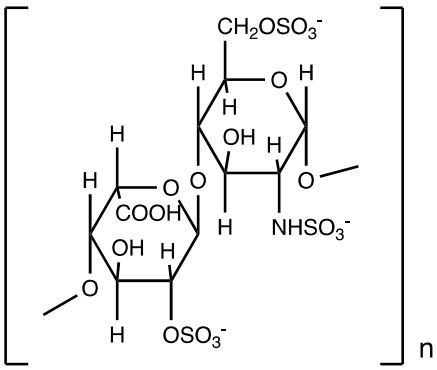

Figure 3. The general structure of Heparin; the 1-4 linked uronic acid-glucosamine disaccharide units.

The focus of this review however, will be on polymeric-based designs, on the assumption that these may most naturally mimic and efficiently bind to the polymeric structure of heparin. More specifically, this review will look at the advantages and disadvantages of natural polymer derivatives of polysaccharides, versus synthetic polymers. Previous research has shown that the major issue in using cationic polymers as drugs leads to toxicity[16], due to the large amount of positive charge that can interrupt cell membranes and interact with blood complement.[17,26] Therefore new advancements must find a way to tackle this, whilst still producing a drug with a high enough positive charge to bind heparin, the most anionic biomolecule.

Polysaccharide derived polymers

The arguments for naturally derived polymers being used as heparin antidotes are primarily based on polysaccharide based drugs showing far less toxicity and better biocompatibility[d] than other highly cationic molecules. In addition, because natural compounds such as sugars are often produced by nature in large quantities, they can be very cheap and widely available starting materials for use in synthesis which are important factors when producing a drug on a large scale. Currently, polysaccharide-derived drugs already have a widespread clinical use. Recent research has led to developments in hyaluronic acid being used in gels prescribed for arthritic joint pain[18], and a wound closing device containing chitin microstructures has also been developed.[19] In recent years, there have been two developments in the area of polysaccharide derived drug candidates in the search to find new heparin antidotes.

After previously researching the use of the polysaccharides chitosan[20] and hydroxypropylcellulose[21] for derivatisation as heparin antidotes, in 2015, the group of B. Kalaska *et al*. synthesized a Dextran-based drug, DEX40-GTMAC3. This showed good neutralising ability *in vitro*[e], with a 1.6 ratio of DEX40-GTMAC3 to heparin required for neutralisation.[22] *In vivo*[f] aPTT[g] tests were also carried out which showed that in the case of an overdose (administration of 900U/kg UFH, three times the usual dose) the polymer was able to completely neutralise heparin at the larger required dose of 22.5mg/kg.[22] Although this characteristic of complete effectiveness of the drug at overdose levels of heparin is important, it was suggested that at such high levels of DEX40-GTMAC3, the large concentration of positive charge injected into the blood may cause anticoagulation effects[22], much like protamine. Therefore, careful administration of both heparin and DEX40-GTMAC3, and sensitive detection of heparin levels would still be required. During safety testing, it was found that administration of the drug after heparin injection caused a decrease in blood pressure in rats in both protamine and DEX40-GTMAC3, and on inspection of the results it appears

slightly more so in the latter.[22] Although the decrease was said to be temporary, this may still be problematic, as one safety concern of protamine is its cause of hypotension in some patients. At the dose of 22.5mg/kg, the drug was shown to be extremely hypotensive, showing further the importance of strict heparin level sensing in the blood during surgeries, to avoid complications later on. This drug was deemed very promising at the clinical dose range however, and further studies were conducted to assess the toxicokinetic profile of the drug.[23] Fluorescein-labelling[h] of the drug showed safe distribution of DEX40-GTMAC3 in rats, as only trace amounts of the drug were found in important organs such as the pancreas, spleen, brain and lungs. They concluded that the safety profile of DEX40-GTMAC3 in rodents is favourable to that of protamine based on observations such as no immune response, or organ damage being detected.[23] This is therefore a promising advancement in the search for a new heparin antidote, due to its appealing safety profile and effectiveness in rats at clinical dose (no more than 7.5mg/kg). From this research of DEX40-GTMAC3 it may be argued that polysaccharide drugs are favourable for the design of a new protamine replacement, and this can be seen in their decision to file for a patent in 2011; 'Use of the modified polysaccharides for heparin neutralisation' claiming the rights to using the polysaccharides dextran and hydroxypropylcellulose, modifying them with either positively charged ammonium groups or cationic polymers for use as heparin reversal agents.[24] One of the cationic ammonium groups mentioned by name in this patent was glycidyltrimethylammonium chloride (GTMAC3), which ended up being used in the structure of DEX40-GTMAC3 and was the part of the molecule responsible for binding to heparin. The patent claim was accepted in 2018 and this suggests that polysaccharide derived protamine replacements are a key current development in this area of research.

In the same year, Shagdarova *et al.* synthesized three N-[(2-hydroxy-3-trimethylammonium) propyl] chloride derivatives of chitosan, (Figure 4), derivatives I, II, and III, all of which contained quaternary nitrogen groups responsible for binding to heparin. These derivatives were all quaternised to different degrees; 98%, 40%, and 9% respectively, and derivative I, was found to be the best candidate.[25] Quaternised chitosan derivatives have been shown to have therapeutic use due to their favourable solubility properties over a wide range of pHs, and therefore drugs of this structure may be soluble in all of the various pH environments in the body [26]; derivative I demonstrated a very high solubility at all pHs tested. Overall, the study was minimal and provided little conclusive clinically relevant information on whether this drug would be an effective and non-toxic protamine replacement, although it was determined that from 0.0014-0.0029 mg/mL, derivative I showed neutralisation ability of 0.143 U/mL UFH. [25] This however is only about a third of the usual clinical dosage of UFH, so has limited clinical relevance. Further research by this group in 2017 however, suggested that quaternised chitosan derivatives have cytotoxic[i] effects, possibly due to the positive charge interacting with cell membranes (which have a slightly negative charge), and thus, penetrating cells.[26] It was found that the greater the degree of substitution of the derivatives (i.e., the more quaternisation of the compound), the greater the cytotoxic effect, and therefore derivative I was determined to be far more toxic than derivative III, due to its high degree of substitution.[26] However it was also concluded that the greater the amount of positive charge, the better neutralisation ability of heparin. Therefore, for these compounds to become effective and safe protamine replacements, further structural alterations must be carried out to find the optimal structure consisting of a large enough degree of quaternisation for effective binding to heparin and good solubility, yet low enough to avoid cytotoxicity issues. Many further *in vivo* and *in vitro* tests would then be required for a more rigid proposal of a chitosan-derived drug towards its effectiveness and safety.

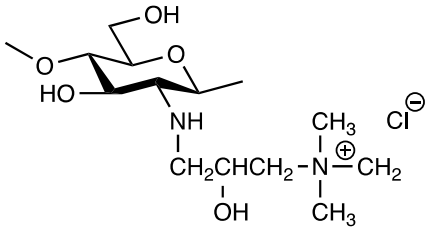

Figure 4. The general structure of the N-[(2-hydroxy-3-trimethylamonium) propyl] chloride derivatives of chitosan.[25]

Synthetic Polymers

An alternative to these naturally derived polymers is synthetic polymers. There is a huge diversity within the types of polymeric structures that can be formed, many of which will be discussed in this section. With far more reagents available for use as starting materials for synthetic polymers, this method may be more beneficial to drug discovery when trying to find a new lead compound, as larger drug libraries may be created.

In 2015, a research group in China consisting of Tong Li *et al*. produced a new synthetic peptide, R15S, which showed good ability to neutralise heparin *in vitro*, demonstrated by the aPTT and anti Xa assay[j] results. These showed that heparin was neutralised fully by R15S at a lower concentration than protamine.[27] In addition, the drug exhibited low cytotoxicity at a dose higher than the clinical dose of protamine, and had a high LD_{50} value[k] of 35.4mgkg^{-1}. Although the clinical dose of R15S would not be found until clinical trials, comparing its LD_{50} to the clinical dosage of protamine, 2-3 mgkg^{-1}, shows a large gap, which is favourable as it dramatically decreases the chances of a lethal dose. Furthermore, there was no indication of the production of antibodies on injection of R15S, [27] and therefore this suggests little risk in patients becoming 'resistant' to the drug, as can occur when protamine is administered to diabetics leading to its ineffectiveness.[28] One drawback to this drug is that, much like protamine, injection of the drug caused a rapid decrease in blood pressure, indicating a risk of hypotension.[27] This side effect is serious, and therefore further alterations to the structure of this peptide would need to be considered before this drug could proceed to clinical trials, to reduce the risk of hypotension to the patient on administration of the drug. In addition, further i*n vivo* tests on the cytotoxicity of the drug including studying the effect of the drug on major organs and its mechanism of excretion should be carried out before this drug could reach clinical trials. Otherwise however, this is a promising attempt for a synthetic peptide protamine replacement.

A pillar[5]arene structure with ten positively charged groups above and below the polymeric ring was synthesised by a group in 2018, with the name P10+, (figure 5).[29]

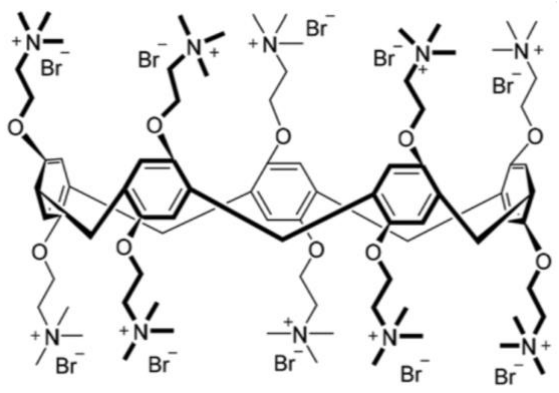

Figure 5. The structure of the pillar[5]arene host, P10+. The positively charged ammonium ions above and below the plane of aromatic rings bind anionic heparin.[29]

This potential drug-molecule acts as a host-guest complex[l], with the aim to provide both a neutralising effect on heparin, and information on the concentration of free heparin in the blood after administration of the antidote, for use during surgeries. The host-guest complex works as an example of an indicator displacement assay.[m] In this case, the host is the P10+ complex, and the guest is methyl orange (MO). This works by the MO guest weakly complexing with the P10+ structure by sitting in the centre of the pillararene ring. This is displaced by heparin which exhibits more favourable interactions with the host due to the 'neutral charge balance' upon binding (Figure 6).[29] This neutralisation effect is a favourable property due to largely positively charged / negatively charged drug molecules leading to toxic side effects in patients[12,26], and therefore may indicate the possibility of a good safety profile of the drug. The resulting UV-Vis absorption spectra on addition of heparin to the host-guest complex showed an increased ratio of the absorbance of free MO over the bound MO, meaning that heparin effectively displaces the MO guest, although data shows that it does not completely displace it.[29] P10+ was also found to have good promise for clinical value; the anti-Xa *in vitro* test found P10+ to be 98% effective in neutralising heparin, and the Dynamic Light Scattering (DLS)[n] studies showed that the P10+-heparin complex was small, [29] thus would further suggest that this drug may be non-toxic as smaller complexes are less likely to activate blood complement which may increase its biocompatibility.[11] This new complex provides promising advances in addressing the issues of inaccurate determination of heparin concentrations in the blood during surgery. To determine whether P10+ could be effective as an antidote to heparin, more tests would need to be run to test its clinical relevance, for example, cytotoxicity and aPTT tests could be carried out to test the safety and effectiveness of the drug *in vivo*. Due to the large amount of positive charge situated on this drug molecule, the drug may be found to have toxicity issues, and further structure alterations may be required to provide a safe protamine replacement.

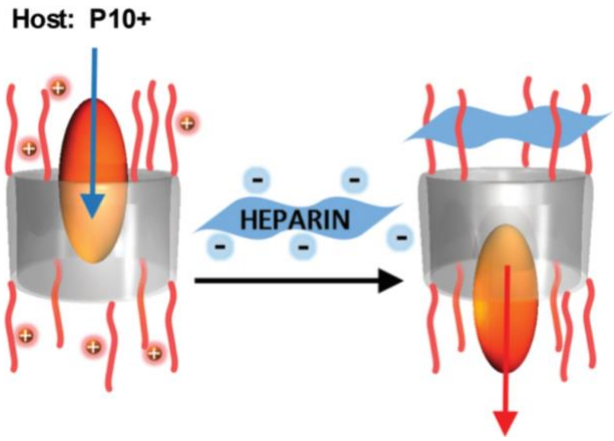

Figure 6. The mechanism of heparin binding causing displacement of the methyl orange guest, leading to increased absorbance of free methyl orange. The positively charged ammonium ions of the host P10+ molecule above and below the plane of aromatic rings favourably bind anionic heparin over methyl orange.[29]

The group of R.A. Shenoi *et al*. developed a dendrimer-type drug with the aim to neutralise all types of heparin, including UFH, LMWH and fondaprinux, called a Universal Heparin Reversal Agent (UHRA) (Figure 7A).[30] Their first study in 2014 led to the finding of UHRA-7. The structure of the UHRA dendrimer consists of a branched polyglycerol core which is surrounded by positively charged 'R' groups named 'Heparin Binding Groups' (HBG, Figure 7B); UHRA-7 contains 20 HBG. Each HBG consists of three quaternary ammonium cations, and these are responsible for the neutralisation of heparin. The 'R' groups are bound to long methoxy-PEGylated chains emanating outwards from the core, forming a 'brush layer' (Figure 7C). This layer shields the positively charged ammonium groups from off-target interactions with other negatively charged molecules including cell membranes and components of the blood, thus increasing its biocompatibility[30]. Alteration of the density of the PEG layer led to the optimum balance between strong enough affinity of the HBGs to bind heparin, and shielding of the cationic charge. Preferential binding of heparin over other biomolecular structures occurs as heparin is the most anionic naturally occurring molecule and therefore will most favourably bind the UHRA. This UHRA structure proved very promising, yielding good results for clinical usage; aPPT and anti-Xa *in vitro* tests showed complete neutralisation of heparin at 0.025mg/mL and showed no sign of anticoagulant effects at the high concentrations of 0.25mg/mL, making this drug beneficial in comparison to protamine.[30] The toxicity of this drug was also suggested to be very low; *in vivo* tests were carried out to determine the safety of this drug in mice, and on injection of 50mg/kg, UHRA-7 showed no signs of adverse side effects. The lethal dose must have been higher than this, whereas the lethal dose of protamine was approximately 30mg/kg. This drug therefore could show a better safety profile when also in comparison to R15S, whose LD_{50} was 35.4mg/kg, and DEX40-GTMAC3, which showed anticoagulation properties and signs of hypotension at such high concentrations. From atomic force microscopy[o] results it was determined that the size of the UHRA-7-UFH complex was much smaller than the protamine-UFH complex (<100nm compared to 100-500nm).[30] The large size of complex has been suggested to be in relation to the toxicity of protamine observed after surgery, due to its activation of complement in blood plasma.[11] When tested, it was found that the UHRA complex had no effect on blood complement, whereas the protamine complex was shown to activate it.[30] This is very beneficial to the UHRA-7 drug, as activation of blood complement may lead to unwanted immune responses which could cause further complications after surgery. In conclusion, UHRA-7 shows promising superior characteristics over the use of protamine sulphate.

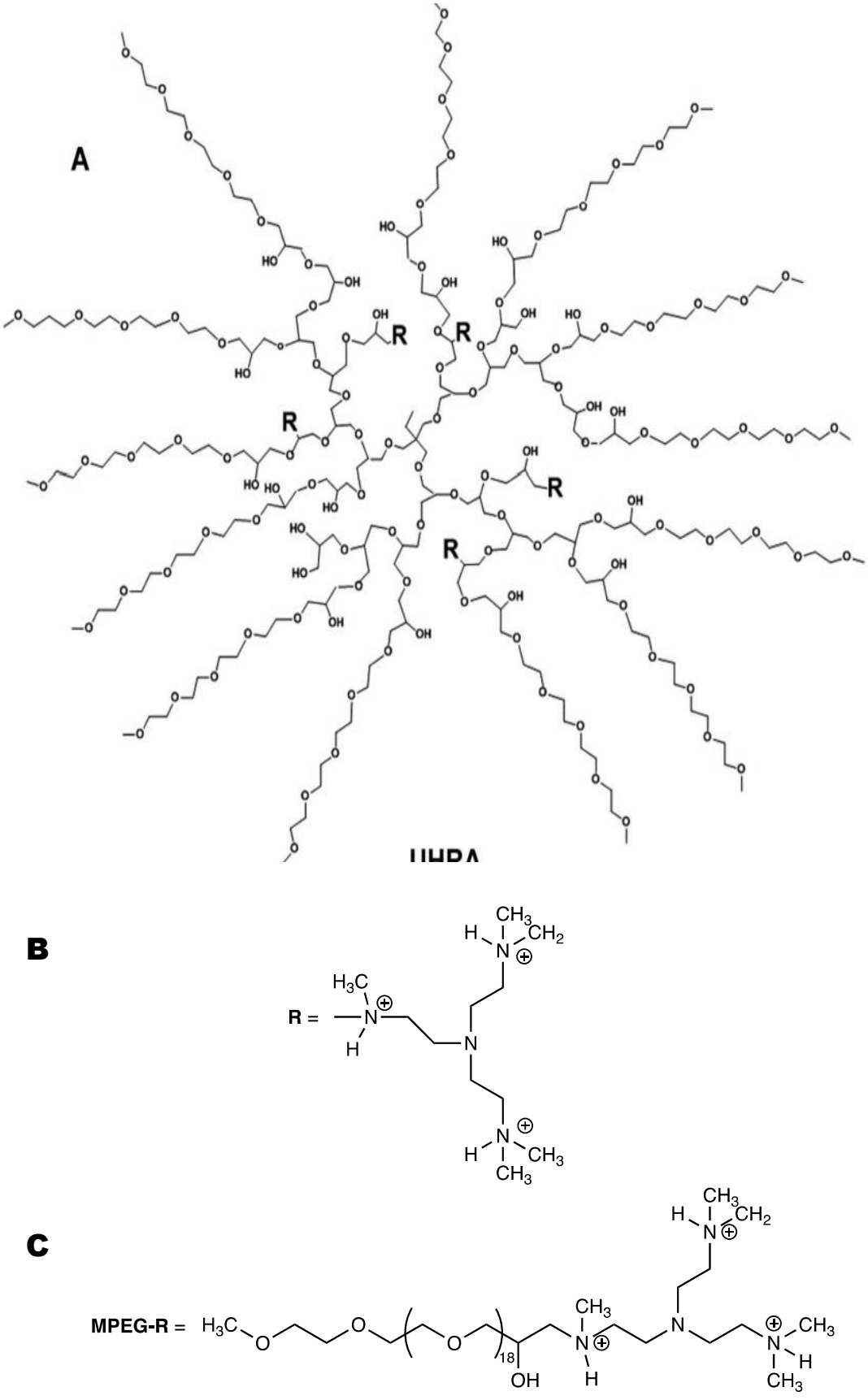

Figure 7. A: The general structure of UHRA; The polyglycerol dendritic core is surrounded by positively charged heparin binding groups, R, which are shielded by methoxy-PEGylated chains.[30] **B**: The positively charged heparin binding groups. **C**: The methoxy-PEGylated chains attached to the heparin binding groups.

This group made further investigations into the structure of the optimal UHRA drug in 2017, undertaking more tests including "the effect on plasma coagulation, clot stability, morphology and organ toxicity".[31] It was shown by isothermal titration calorimetry[p] tests (ITC) that there are no interactions between UHRA and fibrinogen[q], and therefore the structure of blood clots is not affected by UHRA, as is the case with protamine, which weakens blood clots and is believed to be the reason excess bleeding can occur after protamine administration.[31] This is supportive evidence of good safety of the drug. In addition, when excess UHRA levels were administered to mice *in vivo*, the results further supported the *in vitro* findings that the drug does not induce an anticoagulant effect, and there were also no signs of adverse effects on the mice' lungs.[31] UHRA therefore, is a good proposal for a protamine replacement as it appears both effective and safe. After thorough initial testing in the preclinical phases, the next step for this drug would be clinical trials to determine the effectiveness, side effects, and dosage on humans. Shenoi *et al*. have provided, in the UHRA molecule, a sophisticated synthetic way of making a highly cationic drug biocompatible and non-toxic by use of methoxy-PEGylated chains and careful control of density, without the need for using a natural polysaccharide derivative to acquire these desirable characteristics. The ability of the group to make a library of UHRA molecules with a large range of characteristics is an advantage for synthetic polymers, as there are many more possibilities for structural changes so there are higher chances of finding a lead compound early on in the drug discovery process.

In most recent years, block copolymers[r] have become a big area of research into finding a protamine replacement. Block copolymers may have beneficial effects as drugs because joining polymers of different structures together can lead to the formation of a product with a combination beneficial properties from both of the polymers. One big advancement in increasing the biocompatibility of a drug is the introduction of a PEG (poly(ethylene glycol)) group, as in the above example of UHRA-7. PEGylation allows for the administration of a highly positively charged drug to humans whilst lowering the cytotoxic effects caused on cells. This is achieved as the PEG polymer helps to shield the large region of positive charge so that the drug is unreactive towards biological components in the body, such as blood complement.[30,32,33] By avoiding these off-target interactions, a greater percentage of the drug will interact with heparin, thus lowering the side effects and lowering the required therapeutic dose. It was suggested that the PEG block may also reduce the size of the drug-heparin complex, further lowering the toxicity of a drug compared to protamine, as in the case of UHRA-7.[30]

One example of a block copolymer synthesized in Finland, 2016 is PEG_{114}-$PDMAEMA_{52}$. The ability of the compound to bind heparin was analysed by use of an indicator displacement assay using methylene blue. This showed the length of the PDMAEMA block to be important, with longer blocks binding heparin more efficiently, whilst the PEG block had little effect on the binding.[32] Although the reasoning behind the compounds containing longer cationic blocks being more effective was not deduced, it could be suggested that it was due to a larger number of cationic groups leading to more electrostatic interactions with heparin and thus a greater neutralising effect. Another explanation for this could be that longer chains are less shielded by the PEG groups and can extend past the protective layer to form stronger interactions with heparin. DLS measurements provided insight into the size of the PEG_{114}-$PDMAEMA_{52}$-heparin complex formed, which was found to be less than 100 nm, and therefore much like UHRA-7 and P10+, is of favourable size. The toxicity of PEG_{114}-$PDMAEMA_{52}$ was shown to be promising as MTT assays[s] showed that cells mixed with the drug-heparin complexes were still healthy and functioning after 24 hours, although only at low concentrations of the

complexes.[32] The antidote ability of the drug was analysed by the anti-Xa *in vitro* assay. However, results showed that protamine was much more effective at neutralising heparin, with protamine reaching approximately 95% neutralisation compared to about 25% for PEG_{114}-$PDMAEMA_{52}$ at the same concentration.[32] Thus, to proceed with the development of this drug, important structural alterations would be required for this copolymer to have significant clinical relevance, and to make it favourable over protamine.

In the same year, the Polish group of B. Kalaska *et al*. who were previously committed to finding a naturally-derived solution to the protamine replacement issue, diverted their attention also to the PEGylated block copolymer approach, with their attempt at a new drug $PEG_{41}PMAPTAC_{53}$, named 'heparin binding copolymer' (HBC).[33] The general structure can be seen in Figure 8.

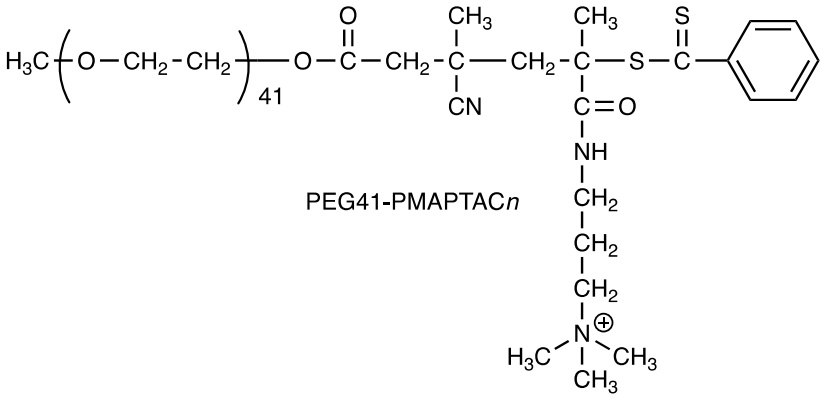

Figure 8. The general structure of $PEG_{41}PMAPTAC_{53}$. The neutral PEG block consisting of 41 monomers is linked to the cationic PMAPTAC group.[33]

In vitro tests showed complete neutralisation of heparin and reversal of the effect of the anticoagulant. When tested *in vivo* using rats, full neutralisation of UFH (300 U/kg) occurred at a within clinical range dose for protamine of 1.95mg/kg, without affecting parameters such as blood count.[33] Further safety of the drug was tested with good results being obtained for the effect on vital organs, as there was no detection of the drug in the heart, lungs, spleen, or brain.[33] The effect of HBC on fibroblast cells[t], and more specifically the effect of PEGylation was also examined; HBC had minimal toxic effect on the cells, with the percentage viability of the cells between all concentrations of the drug administered between 0-150 μg/mL being almost 100%. In contrast, adding the polymer with no PEGylated chains caused significant decreases of cell viability to 40% and lower from concentrations of 50 μg/mL upwards.[33] This is suggestive of both the safety of this drug *in vivo*, and also the beneficial effects of use of PEG blocks in these drugs. In addition to this there were no signs of a decrease in blood pressure as happens with protamine, further indicating the safety of the drug. Other positive aspects of the design of this drug are the inexpensive and easy method of synthesis, which would be reasonable on a large scale. Clearly, this drug resonated as promising within the research group, as they filed for a patent several months later in June 2016, claiming the use of PEG-PMAPTAC polymers for heparin neutralisation.[34]

Alternatives

However, there are other possibilities beyond the scope of polymeric based drugs for forming a potential heparin antidote. Some of the polymers discussed above, for example, the dendritic UHRA molecule, may have a complicated synthesis which could be unrealistic when producing the drug on a large scale if it were to be approved. To overcome this obstacle, other non-polymeric type drugs can be synthesised, including the attempt of V. M. P. Vieira *et al*. in 2017 of a self-assembled cationic micelle, C16-DAPMA.[35] The drug was found to be effective at binding to heparin on full displacement of the dye 'mallard blue' *in vitro*. In addition, it was found that micelles were formed before the CMC value[ɯ] on binding to heparin[35], which may give it favourable qualities as a drug, leading to the possibility of a low therapeutic dose. DLS measurements of the C16-DAPMA-UFH complex were taken; these however did not show promising results, as the complex formed was of great size, 1185nm[35], approximately ten times the size of the complexes formed for UHRA-7 and PEG_{114}-$PDMAEMA_{52}$, which are of favourable size, as it has already been suggested that smaller complexes lead to lower toxicity issues.[11] This complex, is in fact more than twice the size of the protamine-UFH complex, (100-500nm), which exhibits toxicity after administration during surgeries. This paper was mostly focused on the structure and binding of the potential drug to heparin, and therefore, many *in vivo* and safety tests must be carried out to see if the molecule shows any promise as a drug, although it may be likely that toxicity issues will arise based on the size of the complex formed on binding to heparin.

Finally, the Polish group of B. Kalaska *et al*., one of the leading groups for research in this area, have presented a completely different approach to the heparin antidote issue. As mentioned in the introduction, heparin itself is a large part of the problem; its structure is undefined and therefore synthesising a target for the molecule is problematic, and administration of heparin leads to uncertainty in the exact dose active in the blood.[12-13] The large anionic nature of heparin often leads to the production of drug molecules that are highly cationic, resulting in cytotoxicity issues as the positive charge upsets the balance of charge in cell membranes, and interacts with components of the blood.[16,17,26] Therefore, this group have presented an idea, in which they have proposed a new anticoagulant-antidote pair altogether, thus potentially replacing both heparin and protamine. The anticoagulant is a block copolymer, containing a neutral PEG block and an anionic PAMPS block (poly(sodium 2-acrylamido-2-methylpropanesulfonate)) called PEG47-*b*-PAMPS108[36], and the antidote is HBC as discussed above. PEG47-*b*-PAMPS108 showed anticoagulant activity *in vitro* at 1.00 mg/mL during aPTT as it increased the time for coagulation to occur, however the same level of inhibition was reached by UFH at only 0.05mg/mL, and therefore further alterations to the structure may have to be carried out for this drug to be potent enough for clinical use.[36]

One concern of this drug is that it was determined that PEG47-*b*-PAMPS108's mode of action as an anticoagulant was different to that of heparin, as it does not bind to thrombin or factor Xa.[36] This may be the cause of inefficient anticoagulant activity in the drug, as "the accelerated inhibition of thrombin by AT occurs through the ternary heparin-AT-thrombin complex".[3] In addition, it is unable to bind and inhibit as many factors in the coagulation cascade, and therefore, cannot prevent coagulation at as many points in the cascade as heparin, which may also play a role in the less efficient anticoagulant activity of PEG47-*b*-PAMPS108. Further tests may be required to determine whether this alternate mechanism is detrimental to the anticoagulation process. Safety tests were carried out *in vivo* in rats, with the observations that there was no effect on heart rate, respiratory rate, or other cardiorespiratory parameters,[36] however additional testing including toxicity tests and the effect on organs must be carried out before the drug could proceed to clinical trials. Once this anticoagulant had been tested, the

effect of the antidote, HBC, on PEG47-*b*-PAMPS108 was determined by undertaking a number of tests. ITC measurements showed that HBC has a great affinity towards PEG47-*b*-PAMPS108 and binds effectively.[36] DLS measurements showed the HBC-PEG47-*b*-PAMPS108 complex to be small (100nm) which, as above with UHRA-7, has been stated to be a favourable size to avoid toxicity issues.
This advancement in the field is a promising new answer to the heparin-antidote issue, although it is in its early stages of testing, and the structure of the anticoagulant may require adjusting to make it at least as effective as UFH. Further research into this pairing is awaited.

Conclusion

In conclusion, it may be argued that synthetic polymers are a favourable route for the synthesis of a new heparin antidote over naturally derived drugs. Although polysaccharides have been used for many years for medicinal applications due to their high biocompatibility and low cytotoxic effects, it appears that through the synthetic route, which previously led to toxicity issues due to the large amount of cationic charge being placed into the body, there is now a great strategy to overcome this problem; fusing PEGylated chains to the cationic polymer. UHRA and the block co-polymer drug $PEG_{41}PMAPTAC_{53}$ have both shown great ability in the effective neutralisation of heparin, and appear to present promising safety profiles. The volume of new synthetic polymers in the latest years of research compared to naturally derived molecules is far greater, and further suggests their superiority. Even the group including B. Kalaska, K. Kaminski and M. Nowakowska, after spending many years in the field developing the Dextran-based DEX40-GTMAC3, which seemed to display all the positive qualities of a good protamine replacement, stated, “the inherent drawbacks of natural polymers such as high dispersity of the molecular weight, batch-to-batch and source-to-source variability, and difficult purification procedures directed the research on polymeric anticoagulants toward synthetic polymers”.[36] It was also concluded however, that the polymeric approach may lead to difficult synthetic procedures, such as with the dendrimer of UHRA-7, and self-assembled methods such as C16-DAPMA may be beneficial. Future research may lead to advances in developing a new anticoagulant as well as a new antidote, as demonstrated in the above example of PEG47-*b*-PAMPS108 and HBC. Additionally, molecules containing PEGylated chains should be continually researched and integrated into these potential-drug compounds due to their favourable properties. Overall, there have been some very promising advancements in this field of research in recent years, and results from clinical trials of these drugs are awaited.

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