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# Small Heparin Fragments Regulate the Amplification Pathway of Complement

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**Abstract:** Heparin is a highly sulfated, polydisperse and heterogeneous glycosaminoglycan which has been well characterized for its ability to regulate multiple sites in the complement cascade. Although previous studies demonstrated the relationship between degree of sulfation, particularly O-sulfation, and complement inhibiting capacity, they left unclear the relationship between the size of the heparin molecule and its ability to inhibit complement. Therefore, although the structure-activity relationship for heparin is well understood for anticoagulant activity, it is ill defined for the complement system. The present studies were designed to examine depolymerized heparin to determine which fragments were capable of inhibiting amplification pathway activation. We found that as the size of the molecule increases the ability to regulate complement increases; below 1000 Da the fragments were essentially inactive and above 3500 Da they had the same activity as does commercial heparin. Furthermore, we examined the five major tetrasaccharides of heparin and found that the degree of sulfation did correlate with the ability to inhibit complement. These studies have for the first time begun to examine the minimal structural requirements for heparin to regulate complement.

**Key words:** Heparin; Complement; Polyion; Glycosaminoglycan

## Introduction

Heparin is a polydisperse, sulfated copolymer of glucosamine and uronic acid which has been used for the last half century as an anticoagulant (Jaques, 1979). Commercial heparin, with an average molecular weight of 10000–14000, also possesses a multiplicity of other biological activities (Engleberg, 1977; Salzman et al., 1980; Steinbuch, 1982; Folkman et al., 1983) including an ability to regulate complement activation (Steinbuch, 1982). Heparin was first shown to regulate complement activation by Ecker and Gross (1929). Subsequently, multiple sites have been described at which commercial glycosaminoglycan heparin may control the classical pathway (Rent et al., 1976; Raepple et al., 1976; Loos et al., 1976a; 1976b; Caughman et al., 1982). More recently, both fluid phase native proteoglycan heparin and commercial glycosaminoglycan heparin have been shown to be capable of modulating the activation of the alternative-amplification pathway (Weiler et al., 1978). Heparin inhibits generation of cell-bound amplification pathway C3 convertases, C3b,Bb, C3b,Bb,P and C3b,Bb,Nef, by interfering with the binding site on C3b for B (Weiler et al., 1978). Furthermore, heparin prevents fluid phase consumption of B by D in

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*Abbreviations:* GVB, half-isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin; $DGVB^{++}$, GVB containing 2.5% dextrose, 0.5 mM magnesium and 0.15 mM calcium; NMR, nuclear magnetic resonance; HPLC, high pressure liquid chromatography.

the presence of C3b, again indicating a direct action on C3b (Weiler et al., 1978).

The ability of heparin to inhibit the alternative-amplification pathway of complement has been shown to be independent of its antithrombin binding activity and to require the presence of O-sulfation and N-substitution but not N-sulfation (Cofrancesco et al., 1979; Kazatchkine et al., 1981). These previous studies left unclear, however, the relationship between the size and total charge on the heparin molecule and its ability to inhibit complement activation. Therefore, although the structure-activity relationship for heparin is well understood for anticoagulant activity (Rosenberg and Lam, 1979; Lindahl et al., 1979) it is poorly understood for its effect on the complement system. The present study was designed to explore the structure-activity relationship for heparin's effect on complement. We depolymerized commercial heparin with bacterial heparinase, separated the fractions on the basis of size and charge and studied them for ability to inhibit complement activation. In these studies, we examined heparins of various weights (MW = 4200–20200) as well as heparin fragments (MW = 650–3600) smaller than any low molecular weight heparins that had previously been examined (Kazatchkine et al., 1981).

## Materials and Methods

### *Fractionation of heparin*

Commercial heparin (sodium salt) from porcine mucosa (145 units/mg) was obtained from Hepar Industries (Franklin, OH) and was fractionated on a 125 cm × 1.23 cm Fractogel TSK F55 low pressure column which had a void volume of 77.5 ml and a total volume of 223 ml as determined by blue dextran and sodium azide (Sigma Chemical Co., St. Louis, MO). 200 mg of heparin in 2.0 ml was loaded onto this column and was eluted with 1 M sodium chloride at a flow rate of 0.3 ml/min; it was detected using an Azure A (Fisher Scientific, Fairlawn, NJ) assay (Grant et al., 1984). Seven fractions were collected, desalted using 1000 MW-cutoff dialysis tubing (Catalog Number 1322638, Spectropore, Los Angeles, CA), freeze-dried and reapplied to the column. Four of these seven fractions, which eluted at 111, 122, 124 and 132 ml, were collected, desalted by dialysis and freeze-dried.

### *Preparation and characterization of heparin fragments*

Heparinase was prepared fermentatively from *Flavobacterium heparinum* (Galliher et al., 1981) and separated from contaminating activities as previously described (Linhardt et al., 1984; Yang et al., 1985). Heparin from porcine mucosa was completely digested by treating it with heparinase in acetate buffer (Linhardt et al., 1982), after which the mixture was acidified to pH 4 and the enzyme removed by passage over a 2.5 cm × 0.5 cm column containing SP-Sephadex C-50 (Pharmacia Fine Chemicals, Piscataway, NJ). The eluate, containing the mixture of heparin fragments, was adjusted to pH 7, freeze-dried and size fractionated (Grant et al., 1984) by passage over columns containing Fractogel TSK F50F (17 cm × 2.5 cm) and TSK 40F (80 cm × 2.5 cm) (MCB Manufacturing Chemists, Gibbstown, NJ) run in series. Sodium azide and blue dextran were used to measure the void volume (144 ml) and the total volume (447 ml) of the column. The di (270 ml), tetra (231 ml), hexa (212 ml), octa (197 ml), deca (185 ml) and higher oligosaccharides were obtained in fractions as indicated (Grant et al., 1984). The di- and tetrasaccharides were desalted by passage over a column containing Sephadex G10 (Pharmacia Fine Chemicals) and the larger fragments were desalted by dialysis (1000 MW cutoff, Spectropore). Each fraction was then rechromatographed using the same Fractogel columns and again desalted. The disaccharide fraction consisted of a single component and thus was not further purified. The tetrasaccharide mixture was applied to a Partisil PXS 10/25 SAX analytical high pressure liquid chromatography (HPLC) column (Whatman, Clifton, NJ) and eluted at a flow rate of 2 ml/min using a 0.2–1.0 M linear sodium chloride gradient at pH 3.5 and the five major peaks were collected. Reapplication of each individual tetrasaccharide resulted in a single peak.

*Characterization of heparin and its fragments*

The average molecular weight of heparin and heparin fragments was determined by HPLC size-exclusion chromatography using a 50 cm × 7.5 mm TSK-Gel G2000SW column fitted with a 10 cm guard column (Toyo Soda, Tokyo, Japan) which was eluted with 0.5 M sodium chloride at a flow rate of 2 ml/min. The molecular weight of heparin fragments was also examined using the absorbance at 232 nm of their $\Delta$ 4,5-unsaturated nonreducing ends at fixed concentrations; we assumed the same molar absorptivity as has been reported for the disaccharide fragment ($\varepsilon = 5.42 \times 10^3\ M^{-1}$ (Linker and Hovingh, 1972)). Electrophoresis was performed on 2.5 × 30 cm paper strips in 0.1 M formic acid-pyridine at pH 3.0 for 50 min at 800 V in a Beckman Durran Type R Series D cell. Visualization was accomplished using alkaline silver nitrate (Trevelyan et al., 1950). Uronic acid composition was determined by carbazole (Bitter and Muir, 1962). Chondroitin disaccharide standards were obtained from Miles Biochemicals (Elkhart, IN). Nuclear magnetic resonance (NMR) spectrometry was performed in $D_2O$ on a Bruker WM 360 NMR spectrometer. The precise structure of the disaccharide and the individual tetrasaccharides have been established by various chemical, spectroscopic and electrophoretic methods (Linker and Hovingh, 1984). Anticoagulant and antithrombic activities were measured by activated partial thromboplastin time assay (Linhardt et al., 1982) using Activated Thromboplastin Reagent (Ortho, Raritan, NJ) and by amidolytic assay (Linhardt et al., 1982) using substrate S-2238 (KABI Diagnostics, Stockholm, Sweden) and pure bovine thrombin and antithrombin (generously provided by Professor Whyte Owen of the University of Iowa).

*Complement buffers, complement components and assays*

Half-isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin (GVB), GVB containing 2.5% dextrose, 0.5 mM magnesium and 0.15 mM calcium ($DGVB^{++}$) and GVB containing 40 mM EDTA were used as buffers in the hemolytic assays.

C3 (Tack and Prahl, 1976), B (Hunsicker et al., 1973), D (Fearon and Austen, 1975) and P (Fearon and Austen, 1977) were purified to homogeneity and quantitated as described. C3b was generated from purified C3 as described previously (Gitlin et al., 1975; Weiler et al., 1978). Rat serum, as a source of terminal components, was obtained from Rockland Inc. (Gilbertsville, PA).

EAC4b,3b cellular intermediates were prepared as described previously (Fearon et al., 1973; Lachmann and Hobart, 1978). Heparin and its fragments were examined for ability to inhibit generation of the amplification pathway convertase as previously described (Weiler et al., 1978): 100 $\mu$l of $DGVB^{++}$ alone or $DGVB^{++}$ containing a heparin or heparin fragment dilution was added to tubes (Falcon 2052, Becton-Dickinson, Oxnard, CA). At time zero, 100 $\mu$l of $DGVB^{++}$ containing a suspension of $1 \times 10^7$ EAC4b,3b, 0.28 ng B, 100 ng P and 10 ng D, was added to each tube. The tubes used for the reagent blank and for 100% lysis contained no B. The mixtures were incubated for 30 min at 30°C with shaking. Then 0.3 ml of a 1:15 dilution of rat serum in 40 mM EDTA was added to each tube and incubation continued for 60 min at 37°C. Saline (1.5 ml) was then added to each tube except that the 100% tube was lysed with 1.5 ml of water in place of the saline. Finally, the contents of the tubes were mixed well, centrifuged and percent lysis and the average number of hemolytic sites was determined.

## Results

Polydisperse commercial heparin with an average molecular weight of 13100 was separated on the basis of size into seven fractions (Fig. 1) and four of these, with average molecular weights ranging from 4200 to 22200, were refractionated (Fig. 2). The polydisperse heparin was also depolymerized using Flavobacterial heparinase to produce a mixture of heparin fragments. This mixture was fractionated into di, tetra, hexa, octa, deca, and higher oligosaccharides (Fig. 3). The molecular weights of

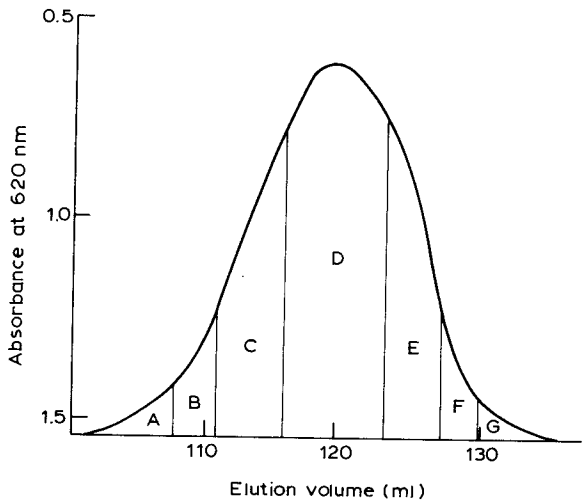



Fig. 1. Low pressure gel permeation chromatography: fractionation of polydisperse porcine mucosal heparin. A–G represent the seven fractions collected.

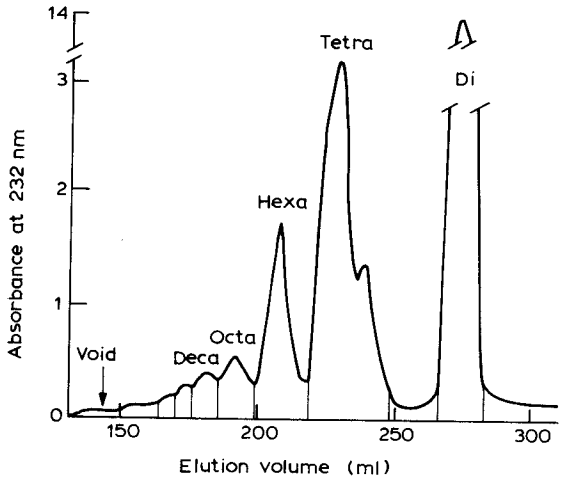



Fig. 3. Low pressure gel permeation chromatography: fractionation of mixed heparin fragments.

heparin and heparin fragments were determined by HPLC size-exclusion chromatography by comparison with standards (Table I). The molecular weights of the heparin fragments were also determined from the absorbance of a known concentration of a fragment as compared to the reported molar absorptivity of the disaccharide fragment (Linker and Hovingh, 1972) and are also given in Table I.

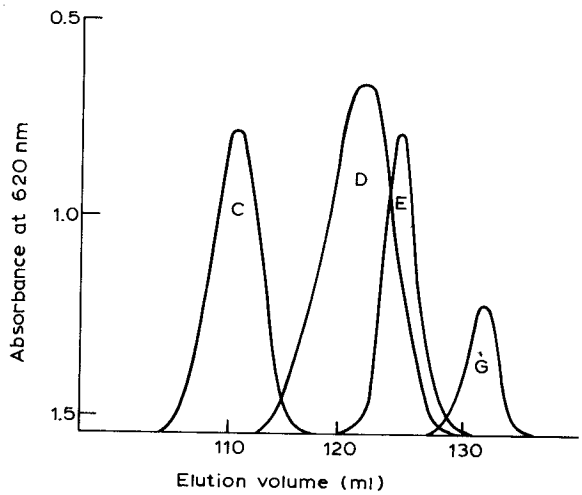



Fig. 2. Low pressure gel permeation chromatography: refractionation of heparin fractions C (high MW), D (intermediate MW), E (low MW) and G (very low MW).

TABLE I

Molecular weight determinations for heparin and its fragments

| Substance | Molecular weight |
|---|---|
| *Heparin* | |
| Polydisperse, commercial | 13 100[a] |
| High MW | 20 200[a] |
| Intermediate MW | 11 100[a] |
| Low MW | 6 100[a] |
| Very low MW | 4 200[a] |
| *Heparin fragments* | |
| Monosaccharide | 221[b] |
| Disaccharide | 665[b] |
| Tetrasaccharide | 1 177[b] |
| Hexasaccharide | 1 400[c,d] |
| Octasaccharide | 2 300[c,d] |
| Decasaccharide | 3 560[d] |

[a] Molecular weight determined by HPLC-gel permeation chromatography using larger fragments ($n$ = 6–14) as standards.
[b] Molecular weight known from established structures.
[c] Molecular weight determined by HPLC-gel permeation chromatography using mono-, di- and tetrasaccharides and standards.
[d] Molecular weight calculated from the absorbance of five known concentrations using the molar absorptivity for the disaccharide ($5.42 \times 10^3$ $M^{-1}$ (Linker and Hovingh, 1972)).

We examined heparin fragments of various sizes at doses ranging from 8 to 0.5 $\mu g/10^7$ EAC4b,3b cellular intermediates for ability to inhibit generation of the amplification pathway. In this experiment, heparin caused 97%, 78% and 30% inhibition at 2, 1 and 0.5 $\mu g/10^7$ EAC4b,3b respectively. The four sized heparin fractions with molecular weights ranging from 4200 to 20200 (Table I) were equally inhibitory with 1.2 $\mu g/10^7$ EAC4b,3b, causing 64, 81, 77 and 77% inhibition respectively. In contrast, the disaccharide (trisulfated disaccharide) caused only 3% inhibition at 4 $\mu g/10^7$ EAC4b,3b (Fig. 4). The amount of inhibition increased with an increase in the size of the heparin fragments (Fig. 4 and Table II). The tetrasaccharides were the smallest fragments that gave a significant amount of inhibition. The largest fragments, a mixture of decasaccharides with an average molecular weight of 3560, were as active as was heparin; 90%, 83% and 48% inhibition was seen at 2, 1 and 0.5 $\mu g/10^7$ EAC4b,3b respectively. The doses which caused 50% inhibition of convertase formation are shown in Table II.

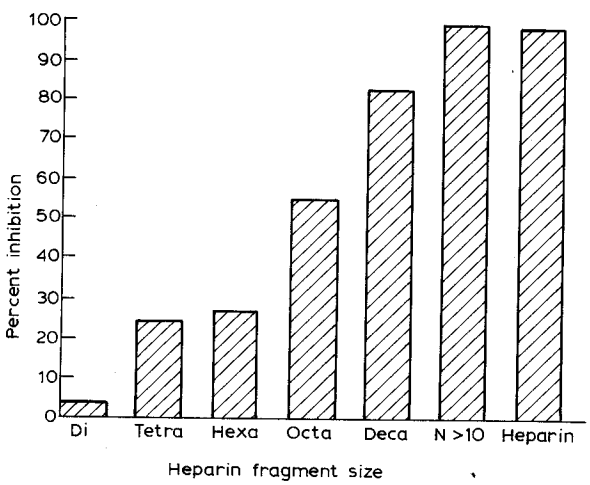



Fig. 4. Relationship between size of the heparin fragment and the amount of inhibition of generation of the amplification pathway of complement. Mixed fragments (disaccharides, tetrasaccharides, hexasaccharides, octasaccharides, decasaccharides and oligosaccharides greater than 10) and commercial heparin were studied at 4 $\mu g/10^7$ EAC4b,3b cellular intermediates and all were inhibitory except for the disaccharide. The amount of inhibition increased with an increase in the size of the fragment.

TABLE II

Concentrations of saccharides causing 50% inhibition of the generation of the EAC4b, 3b, Bb,P convertase

| Saccharide | Concentration ($\mu g/10^8$ EAC43) |
|---|---|
| Disaccharide | >100 |
| Tetrasaccharide (mixed) | 85 |
| Hexasaccharide (mixed) | 75 |
| Octasaccharide (mixed) | 36 |
| Decasaccharide (mixed) | 15 |
| Fragments larger than decasaccharides | 5 |
| Heparin | 7 |

In addition to the mixed oligosaccharide fragments, we examined a trisulfated disaccharide and the five major heparin-derived tetrasaccharides. Strong anion exchange HPLC showed that these five major tetrasaccharides comprised over 60% of the tetrasaccharide fraction. Each was prepared to greater than 95% purity (as assessed by electrophoresis) and the structure established by chemical, spectroscopic and electrophoretic methods. Carbazole assay, using chondroitin disaccharide standards, showed the trisulfated disaccharide to have one uronic acid residue, while each of the five tetrasaccharides had two uronic acid residues, thus confirming their size. The degree of sulfation was established by electrophoresis (Weissmann and Chao, 1981) against chondroitin disaccharide standards. NMR was used to establish the position of the sulfate groups (Gatti et al., 1979) and the nature of the internal uronic acid residue (Huckerby and Nieduszynski, 1982). The structures of the disaccharide and of tetrasaccharides 1 and 2 determined by these methods were in agreement with those previously reported (Linker and Hovingh, 1984).

The five major tetrasaccharides were examined for ability to inhibit complement activation (Fig. 5). The mixed tetrasaccharides were slightly more active than any of the individual components; tetrasaccharide 1 was the most active, tetrasaccharide 3 and tetrasaccharide 5 were less active and tetrasaccharide 2 and tetrasaccharide 4 were essentially

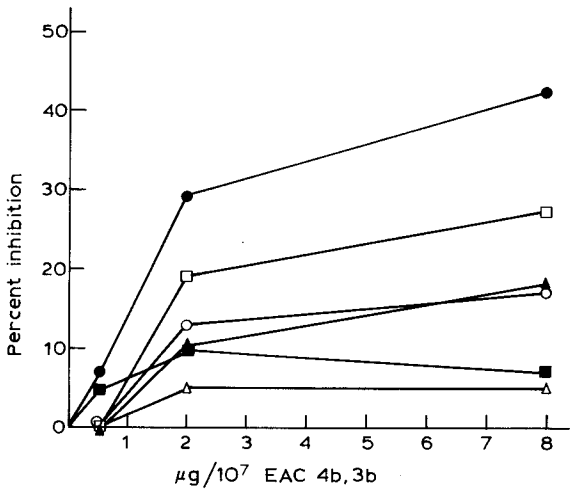



Fig. 5. Comparison of purified preparations of the five major tetrasaccharides (●, mixed tetrasaccharides; □, tetrasaccharide 1; △, tetrasaccharide 2; ○, tetrasaccharide 3; ■, tetrasaccharide 4; and ▲, tetrasaccharide 5) for effects on generation of the amplification pathway of complement.

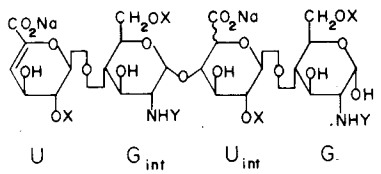
inactive. Of the five tetrasaccharides tested, tetrasaccharide 1 has the highest degree of sulfation (Fig. 6).

## Discussion

This study establishes the relationship between molecular weight and ability of heparin fragments to inhibit complement activation (Fig. 4). There was a progressive increase in the level of complement inhibition as the size of the fragments increased. This parallels the relationship between an increase in anticoagulant activity and an increase in the size of heparin fragments. Tetrasaccharides were the smallest heparin fragments that were capable of exerting inhibition, and fragments with 10 or more saccharides were as active as was commercial heparin itself.

Previous workers in this area did not examine the relationship between the size of the heparin fragment and inhibition because they only considered heparins that were larger than 7000 Da (Cofrancesco et al., 1979; Kazatchkine et al., 1981). Our studies demonstrate that heparins larger than 4200 Da were as active as was unfractionated commercial heparin on a weight basis (Fig. 4).

When we examined the five major individual tetrasaccharide fragments (comprising more than 60% of the tetrasaccharide mixture) we found that one of them was nearly as active as was the mixture containing more than a dozen different tetrasaccharides (Fig. 5). The most active of the tetrasaccharides tested was tetrasaccharide 1, which is also the most highly sulfated of the tetrasaccharides, having six sulfates (Fig. 6). This is consistent with the previous reports that the degree of sulfation (particularly O-sulfation) is important in determining whether a fragment is inhibitory (Kazatchkine et al., 1981). It has been shown that reducing heparin's carboxylate group greatly diminishes the




| Tetrasaccharide | U | $G_{int.}$ | | $U_{int.}$ | | G | |
|---|---|---|---|---|---|---|---|
| | X | X | Y | C5 | X | X | Y |
| 1 | $SO_3Na$ | $SO_3Na$ | $SO_3Na$ | idu | $SO_3Na$ | $SO_3Na$ | $SO_3Na$ |
| 2 | $SO_3Na$ | $SO_3Na$ | $SO_3Na$ | glu | H | $SO_3Na$ | $SO_3Na$ |
| 3 | $SO_3Na$ | H | $SO_3Na$ | glu | H | $SO_3Na$ | $SO_3Na$ |
| 4 | $SO_3Na$ | H | $COCH_3$ | glu | H | $SO_3Na$ | $SO_3Na$ |
| 5 | $SO_3Na$ | H | $COCH_3$ | idu | H | $SO_3Na$ | $SO_3Na$ |

Fig. 6. Structures of tetrasaccharides 1–5.

anticoagulant activity of the molecule but has no effect on its ability to inhibit complement activation (Cofrancesco et al., 1979). None of the five tetrasaccharides we studied possesses any anticoagulant activity by either the activated partial thromboplastin time or thrombin amidolytic assays (Linhardt et al., 1982). Clearly these past studies and the present studies demonstrate that the ability to inhibit coagulation and the ability to regulate complement activation are governed by different properties of the heparin molecule.

These studies are important scientifically because they increase our understanding of the mechanism by which heparin acts on complement activation. They are the first in which structurally defined fragments of heparin have been examined for their ability to inhibit complement. This technology should be useful in identifying additional specific fragments with inhibitory capacity and should begin to define the structure-activity relationship for heparin inhibition of complement activation. It is obvious from these initial experiments that some heparin fragments, such as the disaccharides, are too small to exert inhibition. On the other hand we have already identified at least one small fragment, tetrasaccharide 1, which is capable of inhibiting complement activation without having any effect on coagulation. Future studies will be directed at identifying small fragments (hexa-, octa- or decasaccharides) having activity on complement comparable to heparin but without anticoagulant activity. Such fragments might be clinically useful in controlling complement activation without modifications or in the preparation of heparin analogues. Certainly, the information gained in these studies will lead to a better understanding of the mechanisms by which charged substances regulate the complement cascade.

## Acknowledgements

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