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How to analyze the anticoagulant and antithrombotic mechanisms of action in fucanome and galactanome?

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Abstract Through the perspective of the current glycomics age, fucanomics and galactanomics denote the international projects concerned with the studies of the biomedically active marine sulfated fucose- or galactose-composed polysaccharides, named sulfated fucans (SFs), and sulfated galactans (SGs), respectively. SFs and SGs are isolated from algae or marine invertebrates. The range of therapeutic actions of SFs and SGs is impressively broad. When certain structural requirements are found, some SFs and SGs may exhibit beneficial properties in inflammation, nociception, hemostasis (coagulation and thrombosis), vascular biology (angiogenesis), oncology, oxidative-stress, and virus infections. Although many biomedical applications for SFs and SGs have been pointed out over the past two decades, only inflammation, hemostasis, cancer, and vascular biology have their mechanisms of action satisfactorily elucidated. In addition, advanced structure-function relationships have been achieved only for the anticoagulant and antithrombotic activities, in which glycans of well-defined structures have been assayed. Because of this, the activities of SFs and SGs in stopping the clot and thrombus formation represent the closest therapeutic areas of having these glycans truly explored for drug development. Here, through an analytical viewpoint, we present the common methods and protocols employed to achieve such advanced structure-function relationships of SFs and SGs in anticoagulation and antithrombosis.

Keywords Glycomics · Sulfated fucan · Sulfated galactan · Drug development · Thrombosis

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Abbreviation

ADP	adenosine diphosphate
aPTT	activated partial thromboplastin time
AT	antithrombin
bFGF	basic fibroblast growth factor
HCII	heparin cofactor II
HMWK	high-molecular-weight kininogen
IU	international unit
KccT	kaolin cephalin clotting time
PBS	phosphate-buffered saline
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PT	prothrombin time
PTT	partial thromboplastin time
SFs	sulfated fucans
SGs	sulfated galactans
TCT	thrombin clotting time
TF	tissue factor
TT	thrombin time
UFH	unfractionated heparin
VEGF	vascular endothelial growth factor

Introduction into fucanome and galactanome

Fucanome and galactanome comprise the international projects of glycomics concerned with the functional and structural studies of the sulfated fucose- and galactose-composed polysaccharides named respectively sulfated fucans (SFs) and sulfated galactans (SGs) [1–4]. SFs including the well-known brown algal fucoidans, and SGs including the widely studied red algal carrageenans and agarans, are less commonly known classes of glycans under investigation. Both glycan types can be found in algae (SFs are exclusively from brown

algae, whereas SGs can be found either in green or red algae), and invertebrates such as sea-cucumbers, sea-urchins, and ascidians. The increasing interest on these types of marine glycans over the last 20 years has occurred mostly because of their potential biomedical properties [1–11]. This growing interest was also accompanied by the technological evolution of glycomics, and the appearance of its sub-projects [1, 2]. Some SFs and SGs, essentially those from invertebrates and red algae, exhibit a very rare pattern of well-defined chemical structures (Table 1). This fine structural pattern has been an additional reason for the growing interest of the studies about these glycans. This structural regularity not only makes easier characterization works but also leads to accurate structure-function correlations [2, 4, 6, 7]. The range of therapeutic actions of SFs and SGs are impressively broad. They include benefits in inflammation [7–9], nociception [8], hemostasis (coagulation and thrombosis) [2–7, 9], vascular biology (angiogenesis) [7], oncology [7], oxidative-stress [9], and virus infections [10].

Mechanisms of actions

Despite the several clinical systems in which SFs and SGs have been reported to be active, trustworthy explanations about their molecular mechanisms of action have been achieved only for inflammation, hemostasis, vascular biology, and cancer [2]. In their anti-inflammatory actions, SFs and SGs are capable to interact specifically with P- and L-selectins (curiously not E-selectin) [7], and very likely with certain chemokines that express heparin-binding sites [2]. These inhibiting interactions abrogate the trafficking, activation, and the resultant infiltration process of leukocytes to the inflamed sites. Furthermore, the specificity of interactions with just certain types of selectins collaborates towards selectivity in action of these carbohydrate-based drug candidates.

The anti-angiogenic effect of the SFs and SGs is the main route for their *in vivo* anticancer properties, although some samples have shown the ability to additionally reduce *in vitro* the cell-adhesion capacity of certain highly metastatic cancer cell lines [11]. However, no molecular mechanisms underlying this latter effect have been postulated so far, and perhaps the inhibiting capacity of SFs and SGs on selectins may be involved. Nevertheless, the known mechanisms of action involved in antitumor angiogenesis reside mainly in the inhibition of the basic fibroblast growth factor (bFGF), and the vascular endothelial growth factor (VEGF), either through direct interactions with them or with their specific receptors [7]. The inhibiting process of these pro-angiogenic factors by SFs and SGs disturbs the appropriate balance that feeds the cell differentiation required to supply the neovasculatization. This is crucial for the tumor growth.

Table 1 Few illustrative examples of oligosaccharide repetitive units of the structurally well-defined SFs and SGs. These polysaccharides are extracted from sea-urchins (Echinoidea), sea-cucumber (Holothuroidea), red algae (Rodophyta), and ascidians or tunicates (Ascidiacea)

Species (Class)	Structure
Ludwigothurea grisea (Holothurioidea)	[→3)-α-L-Fuc*p*-2,4di(OSO_3^-)-(1→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$
Strongylocentrotus purpuratus I (Echinoidea)	80 % [→3)-α-L-Fuc*p*-2,4di(OSO_3^-)-(1→]n and 20 % [→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$
Strongylocentrotus purpuratus II (Echinoidea)	[→3)-α-L-Fuc*p*-2,4di(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$
Strongylocentrotus franciscanus (Echinoidea)	[3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$
Strongylocentrotus droebachiensis (Echinoidea)	[→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$
Strongylocentrotus pallidus (Echinoidea)	[→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$
Lytechinus variegatus (Echinoidea)	[→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-2,4di(OSO_3^-)-(1→]$_n$
Arbacia lixula (Echinoidea)	[→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→4)-α-L-Fuc*p*-(1→4)-α-L-Fuc*p*-(1→]$_n$
Echinometra lucunter (Echinoidea)	[→3)-α-L-Gal*p*-2(OSO_3^-)-(1→]$_n$
Glyptosidaris crenularis (Echinoidea)	[→3)-β-D-Gal*p*-2(OSO_3^-)-(1→3)-β-D-Gal*p*-(1→]$_n$
Botryocladia occidentalis (Rodophyta)	[→3)-β-D-Gal*p*-2R_1-4R_2-(1→4)-α-D-Gal*p*-2R_3-3R_4-(1→]$_n$, where $R_{1\text{-}4}$=OSO_3^- or OH, R_1 and R_2=OSO_3− in ~ 66 %, and ~ 33 %, respectively.
Gelidium crinale (Rodophyta)	[→3)-β-D-Gal*p*-2R_1-4R_2-(1→4)-α-D-Gal*p*-2R_3-3R_4-(1→]$_n$, where $R_{1\text{-}4}$=OSO_3^- or OH, R_1 and R_2=OSO_3^- in ~ 60 %, and ~ 15 %, respectively.
Styela plicata (Ascidiacea)	{→4)-α-L-Gal*p*-2[→1)-α-L-Gal*p*]-3(OSO_3^-)-(1→}$_n$
Hedmania monus (Ascidiacea)	[→4)-α-L-Gal*p*-3(OSO_3^-)-(1→]$_n$

In anticoagulation and antithrombosis, certain SFs and SGs can interact directly with serpins (antithrombin, AT, and heparin cofactor II, HCII) as well as with pro-coagulant and prothrombotic proteases, like thrombin (IIa) and factor Xa (FXa) [6]. These molecular interactions very often lead to the generation of ternary complexes (SF- or SG-serpin-protease), which in turn result in the inhibition of the coagulant and thrombotic protease. The duration of these complexes are regulated either by template mechanisms, in which the polysaccharide makes a bridge and bring together both serpin and protease (Fig. 1a), or by allosteric mechanisms (Fig. 1b) in which the structural conformation of the serpins can be conditioned to a higher-affinity-structure onto the protease. Regardless the mechanisms involved, the rate for the serpin-protease complex formation is increased over one order of magnitude in the presence of the exogenous glycan [2, 6].

The special role of the structurally well-defined SFs and SGs

Although some attempts to establish a minimal structure-function relationship in the above-mentioned clinical actions have been made [7, 11], structural determinants of SFs and SGs have been accurately proposed only for their anticoagulant and antithrombotic properties [2–4, 6]. This is like a consequence of two factors. Firstly, hemostasis comprises the mostly studied clinical field for these molecules. This happens because of the pressing need of alternative agents to heparin, together with the elevated demand of new therapeutics in the cardiovascular area due to the high incidence of thromboembolic diseases. Secondly, up-to-now SFs and SGs of well-defined chemical structures (Table 1) have been assayed only in these clinical systems. From these studies, the mechanistic influence of sulfation patterns, monosaccharide types, linkage types, and conformational preferences necessary for the proper bindings with the coagulation cofactors have been pointed out [4]. As a consequence, advanced structure-function relationships have been proposed for these systems [2–4]. These achievements comprise really breakthroughs towards drug development in fucanomics and galactanomics. They can provide reliable information concerning the specific structural requirements of SFs and SGs necessary to reach certain levels of clinical effectiveness [2, 4].

With respect to the activities in inflammation, angiogenesis, and cancer, the brown algal SFs have been the mostly used molecular models in attempts to establish some structure-function correlations. The lack of a clear and regular structural pattern in these algal glycans is the main reason that has impaired some advanced propositions. However, the recognition of species with the highest levels of response has been reported [7, 12]. The minimal explanation regarding structure-function relationship of these algal glycans was the influence of sulfation content in their anti-angiogenic properties [7, 12]. However, conclusions about the contribution of sulfation degrees in biological responses of sulfated polysaccharides may be considered too simple, especially when increased activity is

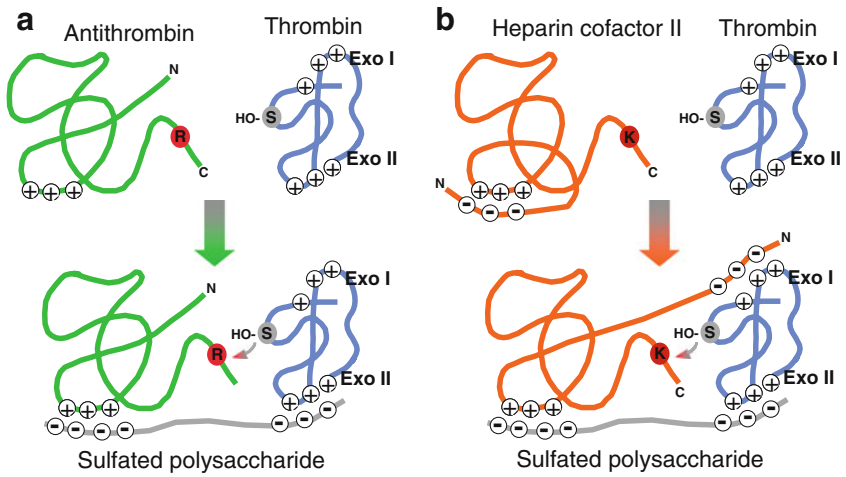

Fig. 1 Molecular schematic representation of (**a**) antithrombin (AT) and (**b**) heparin cofactor II (HCII) activation by anticoagulant sulfated polysaccharides such as the glycosaminoglycan heparin, or SFs and SGs. The thrombin (IIa, in blue) can be inhibited by serpins such as AT (in green) or HCII (in orange). In both cases, the sulfated polysaccharides (SPs, gray line) bring together the serpins and the protease (IIa) mainly through electrostatic interactions of their opposite charges. In the thrombin, this charged cluster is the EXO II. Next, the hydroxyl groups of a serine (S) residue from thrombin will bind to the C-terminus of the serpins, actually to an arginine (R) residue of the AT, or to a lysine (K) residue in the case of the HCII. In the bound-states, a conformational (allosteric) change will occur in both serpins, although this mechanism is more predominant and necessary at the HCII case. Note that the N-terminus of the HCII will interact also with the EXO I of IIa through also electrostatic contacts. With the examples described throughout the text, it's clear that the template mechanism between SPs, serpins (AT, HCII) and protease (IIa) has differential stabilities or formation kinetics directly related to the structural features of the SPs

observed in case of polymers of higher amounts of sulfation content [7, 12]. In addition, the mechanisms in which SFs and SGs exhibit their capacity to inhibit cell-adhesion in metastatic tumor cells are yet poorly understood [11], but speculations over inhibitions of selectins may be raised. Hence, the contribution of fucanomics and galactanomics in inflammation, angiogenesis, and in fights against cancer are still very limited to really push these glycans to drug development in these clinical systems [2].

Based on what has been stated above about the existence of reliable information concerning mostly the anticoagulant and antithrombotic properties of the SFs and SGs, here we will discuss only the methods and protocols commonly used to study the mechanisms of actions in these two therapeutic systems. Furthermore, we will discuss the consequences of the major results obtained using these methods and protocols to achieve advanced structure-function relationships of SFs and SGs regarding their anticoagulant and antithrombotic mechanisms of action. This is particularly possible because of the use of chemically well-defined SFs and SGs (Table 1) in those assays.

Methods and protocols to study the anticoagulant and antithrombotic mechanisms of actions of SFs and SGs

In vitro anticoagulant assays

APTT and the coagulation system

The partial thromboplastin time (PTT) and activated partial thromboplastin time (aPTT or APTT) are perhaps the commonest *in vitro* methods employed to measure the anticoagulation through its end product, the formation of a clot. These methods measure the efficacy of the intrinsic pathway (now widely referred as the contact activation pathway), and the common coagulation pathways. Kaolin cephalin clotting time (KccT) is a historic name for aPTT.

The contact activation pathway begins with formation of the primary complex on collagen by high-molecular-weight kininogen (HMWK), prekallikrein, and FXII (Hageman factor). Prekallikrein is converted to kallikrein, and FXII becomes FXIIa ("a" stands for activated factor). FXIIa converts FXI into FXIa in the coagulation cascade (Fig. 2). Factor XIa activates FIX, which with its co-factor FVIIIa form the intrinsic tenase complex (Fig. 3), which activates FX to FXa. The minor role that the contact activation pathway has in initiating clot formation can be illustrated by the fact that patients with severe deficiencies of FXII, HMWK, and prekallikrein do not have a bleeding disorder. The contact activation system seems to be much more related to inflammation [13].

Although not included in the aPTT assay, the tissue factor (TF) is responsible to generate a thrombin burst, a process by which thrombin (also known as IIa or FIIa), the most important constituent of the coagulation cascade in terms of its feedback activation roles (Fig. 2) is released very rapidly. FVIIa circulates in a higher amount than any other activated coagulation factor. A series of events is seen in the extrinsic pathway, as explained below:

- Following damage to the blood vessel, FVII leaves the circulation and comes into contact with TF expressed on TF-bearing cells (stromal fibroblasts, and leukocytes), forming thus an activated complex (TF-FVIIa).
- TF-FVIIa activates FIX and FX, making thus FIXa and FXa (extrinsic tenase complex, Fig. 3).
- FVII can be also activated by thrombin (FIIa).
- The activation of FX (to form FXa) by TF-FVIIa can be inhibited by tissue factor pathway inhibitor (TFPI).
- FXa and its co-factor FVa form the prothrombinase complex (Fig. 3), which activates prothrombin (FII) to thrombin (FIIa).
- FIIa then activates other components of the coagulation cascade, including FV, FVIII and FXI that activates FIX, which in turn activates FX into FXa.
- FVIIIa is the co-factor of FIXa, and together they form the intrinsic tenase complex (Fig. 3), which activates FX. Through this complex the coagulation cascade is enhanced.

All the complexes mentioned above involve either one of the two main factors of the blood coagulation, thrombin (FIIa) or FXa. These factors are serine proteases. The complexes of the coagulation which involve these serine proteases are vitamin K-dependent and are assembled on the membrane surfaces (Fig. 3).

The *in vitro* aPTT method comprises the measurement of the time course to form a blood clot from a blood sample. From these blood samples, after centrifugation using a simple clinic centrifuge, the plasma, which is used in the aPTT assays, is obtained out from the formed elements of the blood (cells and platelets). The blood samples, prior to centrifugation, are collected from normal patients (without blood coagulation deficiencies) in tubes with oxalate or citrate to arrest calcium. The cation is important to lead the formation of the tenase complexes (Fig. 3). During the aPTT assay, to activate the intrinsic pathway a negatively charged molecule such as phospholipid or another activator, such as silica, celite, kaolin, ellagic acid, and extra calcium amount (to reverse the anticoagulant effect of the citrate and oxalate that have previously robbed the calcium in the blood sample) are mixed into the plasma sample. Then, the time is measured in a coagulation instrument (particularly named coagulometer) until a thrombus (clot) is formed in a cylinder cuvette (usually made of plastic material to avoid the negatively charged silica material of glasses). Usually in the common coagulometers, the thrombus made in the plastic cuvette, which signifies the clotting formation, results in a gel-like structure that strongly holds a steel ball. Then the steel ball starts to circulate at the bottom of

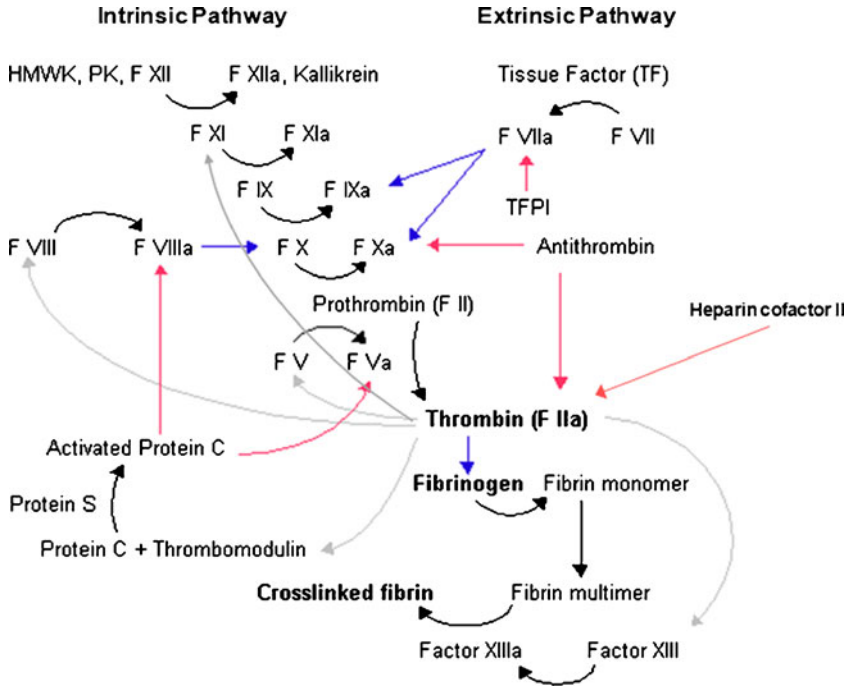

Fig. 2 The intrinsic and extrinsic coagulation pathways. The beginning of the coagulation cascade occurs after a vascular injury and exposure of tissue factor to the blood. This fact triggers the extrinsic pathway of coagulation (right side). The intrinsic pathway (left side) can be triggered *in vitro* when factor XII, prekallikrein, and high-molecular weight kininogen (HMWK) bound to kaolin, glass (silica), or another artificial surface exist. Once bound, reciprocal activation of XII and kallikrein occurs. Factor XIIa triggers clotting via the sequential activation of factors XI, IX and X. The two pathways converge in the formation of thrombin (IIa)

the cuvette. Within the fluid plasma (no clot situation), the ball is held by a magnetic detector at just one side of the cuvette. When the steel ball starts to circulate due to the clot formation, the time measured by the chronometer of the coagulometer stops, signifying the clotting time. The typical reference time range of a control test goes in a range of 30 to 50 s, depending on laboratory and conditions used. In the presence of an anticoagulant agent, the time to form the clot will be prolonged in comparison to the control time (without the anticoagulant agent). Based on what has been state above, the following factors of the blood coagulation are known to be involved for a normal aPTT time: FI (fibrinogen), FII, FV, FVIII, FIX, FX, FXI, and FXII. The test aPTT is termed "partial" due to the absence of tissue factor from the reaction mixture.

The following passage describes a protocol used to measure the aPTT-based anticoagulant activity of some algal and invertebrate SFs. Reproduced with permission [14]. This experimental procedure can actually be undertaken to measure the anticoagulant potency of any agent to be investigated. "Activated partial thrombloplastin time clotting assays were carried out by the method of Anderson *et al*. [15]. Normal human platelet-poor plasma (90 μl) was mixed with 10 μl of a solution of sulfated polysaccharide (0–5 μg) and incubated for 1 min at 37 °C. Then, 100 μl of celite+rabbit phospholipid reagent (Reagent Celite, Biolab, Mérieux) was added to the mixture and incubated for 2 min at 37 °C. Thereafter, 100 μl of 0.25 M $CaCl_2$ was added and the clotting time recorded on a KC4A coagulometer (Heinrich Amelung, Germany). The activity was expressed as international units/mg using a parallel standard curve based on the 4th International Heparin Standard (193 international units/mg)."

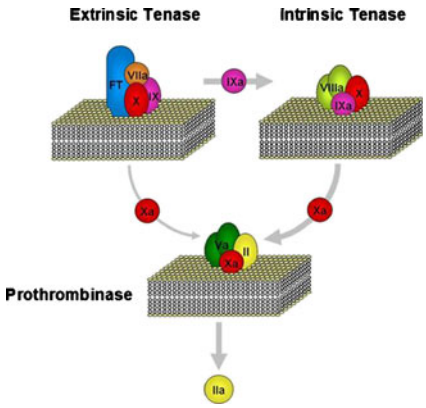

Fig. 3 Schematic representation of the vitamin K-dependent complexes of coagulation. Each serine protease (in red) is shown in association with the appropriate cofactor on the membrane surface

PT

The prothrombin time (PT) is a method to measure the extrinsic pathway of the coagulation (Fig. 2). In terms of methodological procedures it is quite similar to the aPTT assay but, instead of the addition of a negatively charged molecule together with calcium ions; in the PT assay the TF is added. It is worth mentioning that the addition of the different clotting triggers (negative molecule plus Ca^{++} in aPTT, or TF in PT) can measure distinct sides of the coagulation cascade (Figs. 2 and 3). Then in the presence of an anticoagulant agent to be tested, we will be able to distinguish the contribution of its anticoagulant mechanism, either more active at the intrinsic pathway, or more active at the extrinsic pathway, or with equal potencies at both pathways. The following passages, used

from reference [16] with permission, illustrate the protocol used for a PT assay employed for an algal SG. "All coagulation assays (APTT, PT and TT) were performed with a coagulometer, SANCLOT ST (Sanko Junyaku, Co., Tokyo, Japan) as described earlier [17]." "For the PT assay citrated normal human plasma (90 μl) was mixed with 10 μl of a solution of algal extract and incubated for 1 min at 37 °C. Then, PT assay reagent (200 μl), preincubated for 10 min at 37 °C, was added and clotting time was recorded."

TT

The thrombin time (TT), also known as the thrombin clotting time (TCT) is a blood test that measures the time taken for a clot to be formed in the plasma of a blood sample containing anticoagulant agent after an excess of thrombin has been added. It is used to diagnose possible blood coagulation disorders, and to assess the effectiveness of the fibrin formation or fibrinolytic action. The difference in time between a sample tested and the normal time indicates a reduced conversion rate of the fibrinogen (a soluble protein) to fibrin monomers (an insoluble protein), or an accentuated degradation rate of the fibrin molecules (fibrinolytic mechanism). Therefore, changes in the normal time of the TT assay in the presence of an anticoagulant indicate an action of stopping or preventing the formation of fibrin, which is responsible to the last stage of the clot formation (Fig. 2). Normal values for TT are somewhere between 12 and 14 s. TT can be prolonged by anticoagulant polysaccharides such as heparin or anticoagulant SFs and SGs, by fibrin degradation products, and by using plasmas from abnormal patients which are FXIII or fibrinogen deficient.

The following passages used from reference [16] with permission illustrate the protocol used for a PT assay employed for an algal SG. "All coagulation assays (APTT, PT and TT) were performed with a coagulometer, SANCLOT ST (Sanko Junyaku, Co., Tokyo, Japan) as described earlier [17]." "TT assay was performed by the method of Maraganore *et al*. [18] using 4 NIH U ml^{-1} of human thrombin solution. Bovine fibrinogen (0.4 %) and thrombin, instead of human plasma and thrombin, were used throughout anticoagulant purification procedure."

Effect of SFs or SGs on the inactivation of FIIa or FXa by AT or HCII

As described earlier, like heparin, the anticoagulant SFs and SGs have their mechanisms of actions mostly driven by accelerating the inhibiting activity of the main serpins (serine protease inhibitors), AT and HCII, over the two most important proteases of the blood coagulation, FIIa and FXa (see Figs. 1 and 2). As said before, this will result in a ternary-complex, between the sulfated polysaccharide, a serpin and a protease (Fig. 1). The inhibition mechanisms of the sulfated polysaccharides, including the marine SFs and SGs, on these complexes can be measured *in vitro* directly using a mixture of these three purified components incubated together in a system, in which the residual protease, not inhibited by the serpin accelerated by the polysaccharide, can be detected by its catalytic activity (amidolytic activity) towards a specific chromogenic substrate. The proportionally decreasing absorbance of the system with the increasing concentration of the sulfated polysaccharide reflects the rise of the sulfated polysaccharide-catalyzed inhibiting activity on the proteases by the serpins.

For illustrative purpose, Fig. 4 used with permission from reference [19] depicts the resultant curves from the above-mentioned method using the purified coagulation components together with a series of invertebrate SFs and SGs of well-defined chemical structures (Table 1). Based on data represented on this figure, as the concentration of the polysaccharide increases, the residual activities of the proteases Xa and IIa directly measured by decreasing absorbance of the chromogenic substrate indicate the catalytic potential of the analyzed polysaccharide on the system. The glycosaminoglycans heparin and dermatan sulfate are shown with the highest activities on these curves. The IC_{50} values can be obtained individually for each curve (Table 2), and these values are diagnostic of the anticoagulant activity of the investigated polysaccharide.

The following passage, used from reference [19] with permission, illustrates the protocol used for the measurement of the residual amidolytic activity by assaying the purified molecules in a regular spectrophotometer. "Incubations were preformed in disposable semi-microcuvettes. The final concentrations of reactants included 68 nM heparin cofactor II or 50 nM antithrombin, 15 nM thrombin, or factor Xa (all from Diagnostica Stago, Asnières, France) and 0–1,000 μg/ml sulfated polysaccharide in 100 μl 0.02 M Tris–HCl, 0.15 M NaCl, and 1.0 mg/ml polyethylene glycol (pH 7.4) (TS/PEG buffer). Thrombin or factor Xa was added last to initiate the reaction. After 60 s incubation at room temperature, 500 μl 100 μM chromogenic substrate S-2238 for thrombin or S-2222 for factor Xa (Chromogenix AB, Molndal, Sweden) in TS/PEG buffer was added, and the absorbance at 405 nm was recorded for 100 s. The rate of change of absorbance was proportional to the thrombin activity remaining in the incubation. No inhibition occurred in control experiments, in which thrombin was incubated with antithrombin or heparin cofactor II in the absence of sulfated polysaccharide. Nor did inhibition occur when thrombin was incubated with sulfated polysaccharide alone over the range of concentrations tested.

In vivo antithrombotic assays

Antithrombotic polysaccharides can have differential activities on arterial and venous antithrombotic experimental models [20–22]. This is mainly because of the different

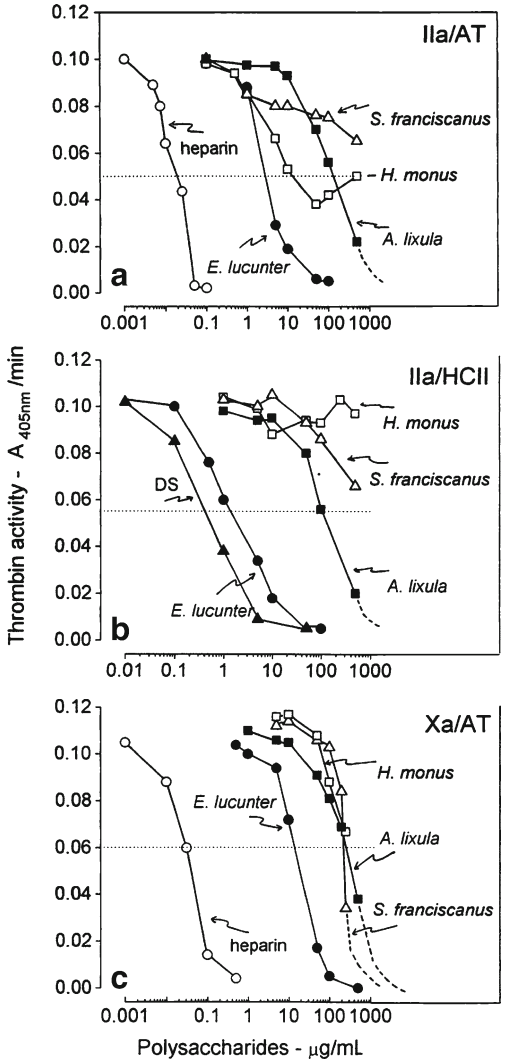

Fig. 4 Dependence on the concentration of 2-*O*- or 3-*O*-sulfated α-L-galactans or α-L-fucans for inactivation of thrombin (**a** and **b**) or factor Xa (**c**) by antithrombin (**a** and **c**) or heparin cofactor II (**b**). Antithrombin (50 nM) or heparin cofactor II (68 nM) were incubated with thrombin (15 nM) or factor Xa (15 nM) in the presence of various concentrations of the sulfated α-L-galactans from *Echinometra lucunter* (*closed circles*) and *Hedmanina monus* (*open squares*), sulfated α-L-fucans from *Arbacia lixula* (*closed squares*) and *Strongylocentrotus franciscanus* (*open triangles*), heparin (*open circles*), and dermatan sulfate (*closed triangles*). After 60 s, the remaining thrombin or factor Xa activity was determined with a chromogenic substrate (A_{405}nm/min). Data reproduced with permission [19]

molecular constitutions of the arteries and vessels. Therefore, when a candidate antithrombotic agent must be assayed, both models have to be tested. Antithrombotic activities are mainly studied by *in vivo* experiments using laboratory rats submitted to a surgery procedure.

Arterial model

For an arterial antithrombotic assay the following passage, extracted from reference [20] with permission, is described: "Carotid artery thrombosis was induced using a modified method of Eitzman *et al.* [23]. Rats (both sexes, ~200 g body weight) were anesthetized with a mixture of ketamine and xylazine, as already described, and secured in the supine position. The right common carotid artery was isolated through a midline cervical incision, and an ultrasonic flow probe (model 0.5 VB; Transonic Systems Inc., Ithaca, NY, USA) was applied. Different concentrations of sulfated polysaccharides were slowly injected into the left carotid and allowed to circulate for 5 min. Rose bengal (90 mg/kg body weight; Fisher Scientific Co., Fair Lawn, NJ, USA) was injected into the inferior vena cava and, immediately after that, a 1.5 mW, 540 nm laser beam (Melles Griot Inc., Carlsbad, CA, USA) was applied to the right carotid artery from a distance of 6 cm and flow in the carotid artery was monitored until complete occlusion occurred."

Venous model

For a venous antithrombotic assay the following passage, extracted from reference [20] with permission is described: "Antithrombotic activity was investigated in rats with rabbit brain thromboplastin as the thrombogenic stimulus [24]. We followed the institutional guidelines for animal care and experimentation. Rats (both sexes, ~200 g body weight, 5 animals per dose) were anesthetized with an intramuscular injection of 100 mg/kg body weight of ketamine (Cristália, São Paulo, Brazil) and 16 mg/kg body weight of xylazine (Bayer AS, São Paulo, Brazil). Different doses of polysaccharides were infused into the right carotid artery and allowed to circulate for 5 min. The inferior vena cava was isolated, and brain thromboplastin (5 mg/kg body weight) from Biolab-Merieux AS (Rio de Janeiro, Brazil) was slowly injected intravenously; after 1 min, 0.7 cm of isolated vena cava was clamped off using distal and proximal sutures. After 20 min stasis, the thrombus formed inside the occluded segment was carefully pulled out, washed with phosphate-buffered saline (PBS), dried for 1 h at 60 °C and weighed. Mean thrombus weight was obtained by the average weight from each group and then expressed as percentages of the weight, 100 % representing absence of any inhibition of thrombosis formation (thrombus weight in the absence of polysaccharide administration)."

Bleeding, platelet aggregation and others

Bleeding Effect test Sometimes, since antithrombotic and anticoagulant agents have the capacity in stopping or impairing the blood clotting, these agents have also, as a downside of their therapeutic uses, bleeding effects. The blood loss in considerable amounts is not a desirable effect for an anticoagulant and antithrombotic drug. Therefore, if a given drug presents this effect real time monitoring is demanded for the

clinical use of this drug. This is the main reason for the continuous monitoring of heparinized patients with unfractionated heparin (UFH) samples. The bleeding side effect is determined in laboratory by *in vivo* experiments also using rats as models.

The following passage, used with permission from reference [20], illustrates the procedural method for bleeding analysis: "Wistar rats (both sexes, ~200 g body weight) were anesthetized with a combination of xylazine and ketamine, as already described. A cannula was inserted into the right carotid artery for administration of different doses of sulfated polysaccharides. After the polysaccharides had circulated for 5 min, the tail was cut 5 mm from the tip and carefully immersed in 40 ml distilled water at room temperature. Blood loss was determined 60 min later by measuring the haemoglobin dissolved in the water using a spectophotometric method, as described by Herbert *et al.* [25]. The volume of blood was deduced from a standard curve based on A_{540nm}."

Platelet aggregation test The platelet aggregation is a step that happens at the latest events of the blood coagulation during the clotting formation. After cross-linked fibrins have made a molecular net that can hold the elements inside the blood vessel, adhesion of both red cells and platelets finishes the clot assemble. During the coagulation, activation of platelets and aggregation help to stabilize the clot. After platelets are activated by inducers such as ADP or thrombin, they become able to aggregate and clump together. Sometimes, anticoagulant and antithrombotic drugs have the additional characteristic of preventing the platelet aggregation, like heparin, which has a clear inhibitory effect on thrombin-induced platelets aggregation [20].

The following passage was extracted with permission from reference [20] to illustrate a protocol for the platelet aggregation assay: "Peripheral venous blood was drawn from human volunteers aged 20–25 years who were non-smokers and had not taken any platelet- active agents during the previous 10 days. Blood was collected into tubes containing 3.8 % sodium citrate and centrifuged at 200×g for 10 min at room temperature to obtain platelet-rich plasma (PRP). An aliquot of PRP was further centrifuged at 1,200×g for 10 min to obtain platelet-poor plasma (PPP). Platelet aggregation in PRP was measured by a turbidimetric method (final platelet count was 300,000/μl) on a Chronolog Aggregometer (Havertown, PA, USA). Baseline (0 %) and 100 % aggregation were established by measuring the light transmission through PRP and PPP, respectively. Sulfated polysaccharides (0–150 μg/ml final concentration) were added to 400 μl of PRP and after 1 min of incubation at 37 °C, ADP (0.1 μM final concentration) was added and aggregation was recorded for 8 min. Controls without GAGs were performed at the same time.

Washed human platelets were obtained from blood anticoagulated with 5 mM EDTA. Platelets were isolated by centrifugation and washed twice with calcium-free Tyrode's buffer, pH 6.5, containing 0.1 % glucose, 0.2 % gelatin, 0.14 M NaCl, 0.3 M $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 2.7 mM KCl, and 0.2 mM EGTA. Washed platelets were resuspended in a modified Tyrode's buffer, pH 7.4, containing 2 mM $CaCl_2$ at 300,000 cells/μl. Assays were performed at 37 °C using a Chronolog Aggregometer (Havertown, PA, USA). Aggregation in the volume of 300 μl was induced by α-thrombin (5 μl of a 60-nM stock solution, 1 nM final concentration). These concentrations of ADP and thrombin were the lowest to induce complete platelet aggregation in the experiments."

Additional experiments Since the effects on the blood coagulation of anticoagulant and antithrombotic agents vary tremendously, like possible pro-coagulant or pro-antithrombotic effects within different concentrations or doses other than those used for the beneficial effects [20], additional assays such as (*ex vivo*) recalcification time, and activation of FXIIa must be undertaken in order to filter out all possible contributions to the hemostatic system. As explained below, the red algal SGs from *Botryocladia occidentalis* and *Gelidium crinale* may present opposite effects depending on the dose used. To avoid superimposition of published materials, and because the main assays and methods concerning the properties on blood coagulation has already been described above, we recommend reference [20] for reading in order to understand these less used experiments.

Advanced structure-function relationships from structurally well-defined SFs and SGs

Monosaccharide type-dependent anticoagulant action

Comparing all the structures illustrated in Table 1, one can discern structural similarities and differences in the SFs and SGs. For example, both SF from *Strongylocentrotus franciscanus* [3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$, and the SG from *Echinometra lucunter* [→3)-α-L-Gal*p*-2(OSO_3^-)-(1→]$_n$ present the same sulfation pattern (exclusive, and entirely 2-sulfated), the same anomeric configuration (α-form), the same glycosidic linkage (1→3) and the same molecular mass (≥100 kDa). The single difference resides in their sugar type (fucopyranose or galactopyranose, respectively) [4]. Interestingly, this single structural difference is itself enough to promote great changes in the anticoagulant properties of these homopolysaccharides (Table 2). The 2-sulfated α-galactan from *E. lucunter* exhibits a significant anticoagulant activity (aPTT of 20 IU mg, although almost 10-fold less than UFH, Table 2). The specific anticoagulant assay with the purified proteases revealed that this SG enhances both FIIa and FXa inhibition by either AT or HCII (Table 2). On the

Table 2 Anticoagulant activities of SFs and SGs measured by aPTT [a], and by IC_{50} for thrombin (IIa) and factor Xa inhibition in the presence of antithrombin (AT) or Heparin Cofactor II (HCII)

Polysaccharide	Species	aPTT (IU/mg)	IC_{50} (μg/mL)		
			IIa/AT	IIa/HCII	Xa/AT
3-linked sulfated α-L-fucans	*S. purpuratus* (I)	76	0.3	0.3	2
	S. purpuratus (II)	10	0.9	2	ND
	S. pallidus	18	>500	>500	>500
	L. variegates	3	>500	>500	>500
	S. franciscanus	~2	>500	>500	250
	L. grisea	<1	>500	>500	>500
4-linked sulfated α-L-fucans	*S. droebachiensis*	<1	ND [b]	ND	ND
	A. lixula	~2	150	150	>500
Sulfated α-L-galactans	*E. lucunter*	20	3	6	20
	H. monus	~2	>500	>500	>500
	S. plicata	<1	>500	>500	>500
Algal SGs	*B. occidentalis*	93	0.02	1.1	2.5
	G. crinale	65	0.02	25	1.5

[a] The activity is expressed as international units/mg using a parallel standard curve based on the International Heparin Standard (193 units/mg)

[b] Not determined

other hand, the anticoagulant effect of 2-sulfated α-fucan from *S. franciscanus* is exclusively based on catalysis of AT inhibition over factor Xa, although it is 12.5-fold less active than the α-SG. This single effect on the Xa/AT system explains the much lower activity of the compound from *S. franciscanus* (aPTT of ~ 2 IU mg-1, 100-fold less active than UFH) since the anti-Xa activity has a relatively minor influence on the aPTT. This is an illustrative and typical example of a sugar-type-dependent biological effect of polysaccharides.

Sulfation pattern-dependent antithrombotic action

Based on this same systematic comparison, the SGs from the red algal species *B. occidentalis,* and *G. crinale* exhibit identical backbones, and chain sizes. However, there are slight differences in their sulfation patterns (Table 1). As a consequence of this difference, the two algal SG differ in their anticoagulant and venous antithrombotic activities, as previously described [20]. SG from *G. crinale* exhibits procoagulant and pro-thrombotic effects in low doses (up to 1.0 mg/kg body weight). At high doses (>1.0 mg/kg), this polysaccharide inhibits both venous and arterial thrombosis in rats. In contrast, SG from *B. occidentalis* is a very potent anticoagulant and antithrombotic compound in low doses (up to 0.5 mg/kg body weight), inhibiting experimental venous thrombosis, but these effects are reverted at high doses. Conversely, arterial thrombosis is only inhibited at high doses (>1.0 mg/kg) of the polysaccharide from *B. occidentalis*. These results indicate that slight differences in the proportions and/or distribution of sulfated residues along the galactosyl chain may be critical for the interaction between proteases, inhibitors, and activators of the coagulation system, resulting in a distinct pattern in the anti- and pro-coagulant activities and in the antithrombotic action. As summarized in Table 2, these structural differences account for the 30 % difference in anticoagulant activity (aPTT) of these algal macromolecules and the even greater difference in catalytic effect of the sulfated polysaccharide on HCII-mediated anti-IIA activity.

Indeed, the structural requirements for the interaction of these marine sulfated polysaccharides with the coagulation cofactors and their target proteases and inhibitors are stereospecific [1–4, 6, 26]. The site of sulfation has a major impact on activity. This can be illustrated by the fact that 2,4-disulfated units have an amplifying effect on the AT-mediated anticoagulant activity in the series of 3-linked α-L-fucans (Table 1, and 2). Specific sulfation sites are required for the interaction with plasma serine protease inhibitors (serpins) like AT and HCII. Note the occurrence of the 4-sulfated unit content in the 3-linked α-L-fucans: *L. variegatus* (a single 4-sulfated unit/tetrasaccharide, Table 1), *S. pallidus* (a double 4-sulfated unit/tetrasaccharide, Table 1), and *S. purpuratus*, isotype II (a double 4-sulfated unit/trisaccharide, Table 1). This 4-sulfation is the structural motif required to enhance the inhibition of IIa by HCII. In contrast, the presence of 2-sulfated residues seems to have a deleterious effect on HCII-mediated anti-IIA activity of the polysaccharides [6].

Conclusions

This document has as its main purpose the description of the major methods and protocols utilized for assaying the properties of SFs and SGs in retarding or stopping the blood clotting and thrombus formation. However, through this description, we have made clear also the importance of the use of the structurally well-defined SFs and SGs in those tests. These

glycans are extracted only from red algal and marine invertebrate organisms such as sea-urchin, sea-cucumber and ascidians. These specific glycans allow a very accurate and advanced structure-function relationship for drug development, especially as anticoagulants and antithrombotics. These are the two main clinical systems in which the particular glycans have been tested. Although, SFs and SGs can also show beneficial effects in vascular biology, cancer biology and inflammation, it is only in hemostasis that these marine compounds of fine structures have demonstrated their real contribution towards drug development. Although certain SFs and SGs are indeed capable to retard the clotting formation, they are still not as potent as heparin as anticoagulant (Table 2). This fact is likely due to the lack of the heparin AT-high affinity pentasaccharide motif in the marine polysaccharides. However, some SFs and SGs can be even better antithrombotic agents than heparin, for example the red algal SGs from *B. occidentalis* [20]. The levels of activity of new polysaccharides on these two therapeutic properties depend intimately on the polysaccharides' structure. In the context of glycomics, fucanomics and galactanomics may comprise two (sub)-projects of promising perspectives. Among all sub-projects of glycomics, fucanome and galactanome may show the best potential benefits to human health care if we consider the most glycans of therapeutic properties described so far. It is worth saying also that although the assays and methods herein described are really efficient to test SFs and SGs, they were not exclusively designed to test solely SFs and SGs. These protocols and experiments can be generally used to any kind of compound wanted to be tested with regards to its anticoagulant and antithrombotic properties.

Conflict of interest Although this document comprises a review in which most of the information sources are coming from already published materials, the use of the current information was totally done under legal procedures regarding the law of copyrights, reprints and permission. The authors state that he is not aware of any authorship, affiliations, memberships, funding, or financial holdings that might be perceived as damaged or as affecting the objectivity of the content of this material. The author declares no conflict of interest by any part.

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