Carbohydrate Polymers 115 (2015) 677–685

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Screening of complex fucoidans from four brown algae species as procoagulant agents

Zhenqing Zhang [a,b,1], Susanne Till [c,*,1], Sabine Knappe [c], Catherine Quinn [a], James Catarello [a], G. Joseph Ray [a], Friedrich Scheiflinger [c,*], Christina M. Szabo [a], Michael Dockal [c]

[a] Baxter Healthcare Corporation, Round Lake, IL, USA
[b] College of Pharmaceutical Sciences, Soochow University, Suzhou, China
[c] Baxter Innovations GmbH, Vienna, Austria

ARTICLE INFO

Article history:
Received 26 June 2014
Received in revised form 26 August 2014
Accepted 1 September 2014
Available online 7 September 2014

Keywords:
Hemostasis
Fucoidan
Hemophilia
NMR
Structure
Screening

ABSTRACT

Fucoidans are complex sulfated polysaccharides extracted from brown algae. Depending on the concentration, they have been shown to stimulate and inhibit blood coagulation *in vitro*. Promotion of coagulation is mediated by blocking tissue factor pathway inhibitor (TFPI). We screened fucoidan extracts from four brown algae species *in vitro* with respect to their potential to improve coagulation in bleeding disorders. The fucoidans' pro- and anticoagulant activities were assessed by global hemostatic and standard clotting assays. Results showed that fucoidans improved coagulation parameters. Some fucoidans also activated the contact pathway of coagulation, an undesired property reported for sulfated glycosaminoglycans. Chemical evaluation of fucoidans' complex and variable structure included molecular weight (*Mw*), polydispersity (polyD), structural heterogeneity, and organic and inorganic impurities. Herewith, we describe a screening strategy that facilitates the identification of crude fucoidan extracts with desired biological and structural properties for improvement of compromised coagulation like in hemophilia.

1. Introduction

Fucoidans are highly complex sulfated polysaccharides extracted mainly from brown algae (Berteau & Mulloy, 2003; Pomin & Mourao, 2008). Apart from mediating antiviral, antioxidant and anticancer effects, fucoidans modulate blood coagulation. In contrast to structurally related heparin, however, fucoidans show anticoagulant activity only at high concentrations (Liu et al., 2006). They were shown to be about 10-fold less active than heparin (Cumashi et al., 2007; Liu et al., 2006; Nishino, Nishioka, Ura, & Nagumo, 1994), having an anticoagulant effect above 100 nM (Liu et al., 2006). Therefore, fucoidans are referred to as non-anticoagulant sulfated polysaccharides (NASPs).

Interestingly, fucoidans also exert procoagulant activity by modulating the activity of TFPI, the major physiological inhibitor of the extrinsic coagulation pathway (Crawley & Lane, 2008). Procoagulant activity occurs at much lower concentrations and fucoidans were thus conceived as a novel class of compounds to improve coagulation (Liu et al., 2006; Prasad et al., 2008). Liu and colleagues determined a procoagulant window of 5 nM to 100 nM, which is in the range of 0.1–10 μg/mL depending on the *Mw*. This procoagulant window was recently confirmed in our laboratory (Zhang et al., 2014).

Fucoidans' procoagulant activity may be useful in treating bleeding disorders such as hemophilia A and B, caused by deficient clotting factors FVIII and FIX, respectively (Lillicrap, 2013). *In vitro* assays have shown fucoidans to reverse the prolonged plasma clotting time induced by TFPI at nanomolar concentrations and to

Abbreviations: NASP, non-anticoagulant sulfated polysaccharide; TFPI, tissue factor pathway inhibitor; TF, tissue factor; s.c., subcutaneous; p.o., per os (oral); *Mw*, molecular weight; polyD, polydispersity; OSCS, over-sulfated chondroitin sulfate; *L.j.*, *Laminaria japonica*; *F.v.*, *Fucus vesiculosus*; *U.p.*, *Undaria pinnatifida*; *E.m.*, *Ecklonia maxima*; CAT, calibrated automated thrombography; aPTT, activated partial thromboplastin time assay; CTI, corn trypsin inhibitor; SEC-MALLS, multi-angle laser light scattering; QELS, quasi-elastic light scattering; dRI, differential refractive index; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; TFA, trifluoroacetic acid; NMR, nuclear magnetic resonance; ICP, inductively coupled plasma; MS, mass spectrometry; AES, atomic emission spectroscopy; dPT, dilute prothrombin time; fl, full-length; *Mn*, number average molecular weight; NOE, nuclear Overhauser enhancement.

* Corresponding authors. Tel.: +43 1 20100 2473410; fax: +43 1 20100 2475733.
E-mail addresses: susanne_till@baxter.com (S. Till), fritz_scheiflinger@baxter.com (F. Scheiflinger).

[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.carbpol.2014.09.001
0144-8617/

accelerate clotting in human hemophilia plasma (Liu et al., 2006; Prasad et al., 2008; Zhang et al., 2014). Procoagulant activity of one fucoidan was demonstrated in bleeding models with hemophilia A mice and dogs after i.v., subcutaneous (s.c.) and oral (p.o.) dosing (Liu et al., 2006; Prasad et al., 2008).

Like most polysaccharides, fucoidans are structurally complex and heterogeneous. Large and disperse *Mw*, complex monosaccharide composition, various sulfation patterns, different linkages and high degree of branching contribute to this complexity (Berteau & Mulloy, 2003; Pomin & Mourao, 2008). Specific structural properties depend on algae species, harvest time, plant parts, location, and extraction procedures (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2010) and influence fucoidans' pro- and anticoagulant activities (Ale, Mikkelsen, & Meyer, 2011; Morya, Kim, & Kim, 2012). Thus, systematic functional and structural assessment is necessary to identify a fucoidan with the desired procoagulant effect. While biological plasma-based test systems for evaluation of fucoidans' hemostatic effects have been established, their structural characterization poses a challenge. A desired fucoidan extract has procoagulant activity without activating the contact pathway of coagulation, is structurally homogenous, of low *Mw* to potentially increase s.c. and p.o. bioavailability, and contains few impurities. Contamination of heparin, one of the few marketed carbohydrate drugs, by oversulfated chondroitin sulfate (OSCS) (Guerrini et al., 2008; Kishimoto et al., 2008), initially went undetected as pharmacopeial methods to monitor the quality of its activity or structure were lacking (Capila & Linhardt, 2002; Linhardt, 1991). Thus, suitable and efficient strategies and methods to characterize fucoidan extracts are critical.

The aim of this study was to functionally and chemically characterize various fucoidan extracts available from a supplier to evaluate the potential of this naturally sourced substance for improvement of hemostasis. In follow-up studies the most suitable material would then be subjected to fractionation and detailed structural characterization (Zhang et al., 2014).

Pre-selection from a set of commercially available fucoidan extracts for their biological activities identified suitable fucoidans from four algae species: *Laminaria japonica* (*L.j.*), *Fucus vesiculosus* (*F.v.*), *Undaria pinnatifida* (*U.p.*) and *Ecklonia maxima* (*E.m.*). We then analyzed in detail their structural properties, impurities and effects on coagulation *in vitro*, thereby applying a systematic approach to identify the most active, highest quality fucoidan extracts (Fig. 1), and developed fucoidan-specific analytical assays.

2. Material and methods

Details are described in Supplemental information.

2.1. Fucoidans and other reagents

We tested fucoidans from four brown algae species: *L.j.* (Baxter Innovations GmbH, Austria), *F.v.*, *U.p.*, and *E.m.* (Marinova, Australia). For *F.v.*, three different lots were included, which were produced from the same seaweed harvest in two independent runs.

L.j. fucoidan was produced by several rounds of ethanol extraction after addition of EDTA. The other fucoidans were extracted by Marinova's proprietary method using dried and milled seaweed. Extraction was achieved by a cold-water process of targeted filtration without using solvents or excipients. All preparations mainly contain sodium cations.

Porcine heparin, alginate (Sigma-Aldrich, Austria) and oversulfated chondroitin sulfate (OSCS, US Pharmacopeia, USA) were included as controls where mentioned. All other chemicals and reagents were analytical grade.

2.2. Calibrated automated thrombography (CAT) assay

Fucoidans' pro- and anticoagulant activities were monitored by CAT developed by Hemker et al. (2003). This assay is based on the measurement of fluorescence generated by cleavage of a fluorogenic substrate by thrombin over time upon initiation of coagulation by tissue factor. Fucoidans were tested at concentrations of 0.02–300 μg/mL in FVIII-inhibited human plasma (Knappe et al., 2013). Their procoagulant effect was assessed by plotting peak thrombin against the concentration within the inclining part of the profile. The EC_{50} was derived using SigmaPlot 12 software from the resulting sigmoidal curve. To evaluate fucoidans' contact activation, CAT assays were performed in normal human plasma with and without corn trypsin inhibitor (CTI)—a specific inhibitor of the contact pathway initiator factor XIIa.

2.3. Activated partial thromboplastin time assay (aPTT)

The aPTT assay is a standard plasma clotting test in which clotting is initiated by a mixture of contact activators and phospholipids. It primarily assesses the intrinsic pathway of coagulation. aPTT was performed in normal human plasma as previously described (Liu et al., 2006). Assays were performed with an ACL Elite Pro instrument (Instrumentation Laboratory, USA). Samples were run in duplicate. aPTT (s) was plotted against fucoidan concentration (0–60 μg/mL); the concentration with a 50% increase in clotting time over baseline was reported.

2.4. Agarose gel analysis

Fucoidans were analyzed by agarose gel electrophoresis (Vieira, Mulloy, & Mourao, 1991; Volpi & Maccari, 2006). Samples (10–20 μg each) were loaded onto a 0.5% agarose gel in 0.04 M barium acetate buffer (pH 5.3) and run for 2 h at 100 mA in 0.05 M 1,3-diaminopropane-acetate (pH adjusted to 9.0 with acetic acid).

2.5. Average Mw and polyD determination using size-exclusion chromatography and multi-angle laser light scattering (SEC-MALLS)

Fucoidans' *Mw* and polydispersities were measured by an Agilent HPLC System coupled with Wyatt Technology DAWN HELEOS, QELS (quasi-elastic light scattering) and Optilab rEX differential refractive index (dRI) detectors. The change in refractive index/concentration (d*n*/d*c*) value (0.113 mL/g) was determined using a *F.v.* sample.

2.6. Monosaccharide analysis using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Fucoidans (2 mg/mL) were hydrolyzed to monosaccharides using 2 M trifluoroacetic acid (TFA) at 100 °C for 4 h. The monosaccharide compositions were analyzed by a Dionex ICS 3000 system (Dionex, USA) equipped with PAD detector, Dionex guard column CarboPac® PA1 (2 × 50 mm), and Dionex analytical column CarboPac® PA1 (4 × 250 mm).

2.7. Fucose, alginate contents and heterogeneity test using ^{13}C nuclear magnetic resonance (NMR)

Quantitative ^{13}C NMR spectra were obtained with a Bruker Avance III 600 NMR spectrometer at a $^{1}H^{13}C$ frequency of 150 MHz with a dual $^{1}H/^{13}C$-cryoprobe.

Alginate content (% mol alginate/mol total polysaccharide) was calculated based on the fact that alginate contains one carbonyl

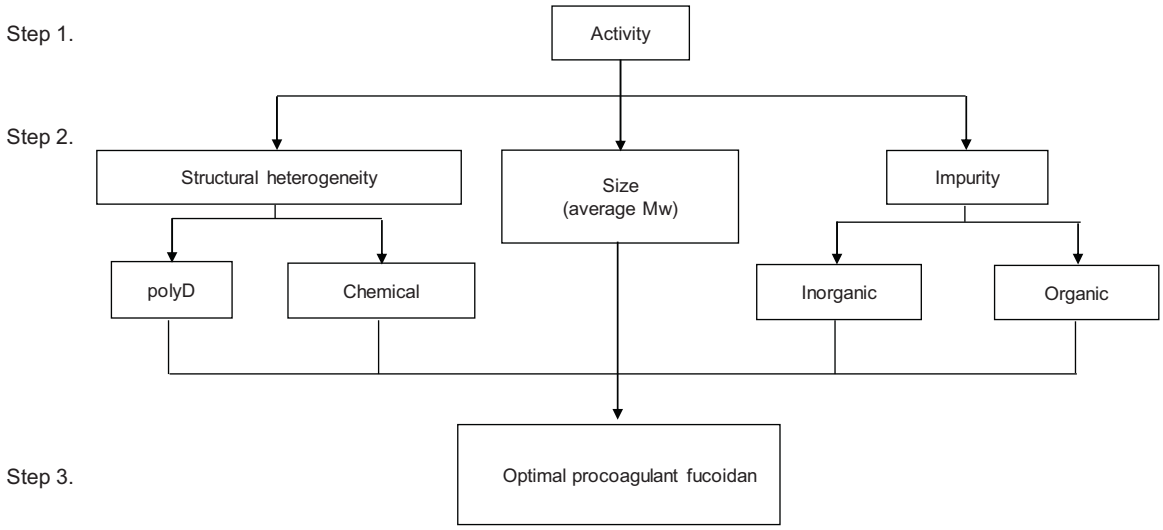

Fig. 1. Flow-chart for screening procoagulant fucoidans.

per saccharide residue, whereas sugar residues of monosaccharides have one anomeric carbon. Therefore:

$$C\%^{\text{alginate}} = \frac{\int \text{carbonyls}}{\int \text{anomerics}} \times 100\% \quad (1)$$

where ∫carbonyls = integral of carbonyl region; ∫anomerics = integral of anomeric region.

Fucose content (% mol fucose/mol fucoidan) was calculated based on the fact that there is one methyl group per fucose residue, excluding the alginate impurity from the total polysaccharide. Therefore:

$$C\%^{\text{fucose}} = \frac{\int \text{methyls}}{\int \text{anomerics} - \int \text{carbonyls}} \times 100\% \quad (2)$$

where ∫methyls = integral of methyl groups.

Eq. (2) was simplified for fucoidan samples with negligible alginate content (<10%):

$$C\%^{\text{fucose}} = \frac{\int \text{methyls}}{\int \text{anomerics}} \times 100\% \quad (3)$$

2.8. Elemental analysis

2.8.1. Inductively coupled plasma (ICP) analyses

The fucoidans' elemental profile was obtained by ICP-mass spectrometry (MS) and ICP-atomic emission spectroscopy (AES).

2.8.2. C, N, H, and S analysis

C, H, and N were measured by PE 2400 CHN analyzer; sulfur was analyzed by colorimetric titration.

2.9. Fucoidan ranking

A ranking system for the most critical screening factors was developed using scores from (1) to (3) or from (1) to (4), with a higher number indicating more desirable properties. EC_{50} values derived from the ascending part of the thrombin peak *vs* fucoidan concentration curve (Fig. 2B) were used to rank procoagulant activity; a score of (3) indicated a fucoidan with the lowest EC_{50}. Ranking of anticoagulant activity was based on the concentration required to increase clotting time of normal plasma by 50%. A high concentration indicates a low anticoagulant effect, which is preferable.

To introduce a parameter reflecting the net effect of intertwined pro- and anticoagulant activity, we calculated the ratio of anticoagulant to procoagulant activity (Table 1). The higher the value, the more the anticoagulant activity is outweighed by the procoagulant effect, and is thus assigned a higher score. A large *Mw*, high polyD and structural heterogeneity is considered less favorable and was assigned a low score. While all kinds of impurities should be considered in judging the quality of fucoidan extracts, we only ranked alginate impurity (Table 4), as all other impurities were negligible or undetected.

3. Results

To identify fucoidan extracts with the desired biological and structural properties, we tested and characterized commercially available fucoidans from four different brown algae species. A flow chart showing the systematic screening process is provided in Fig. 1.

3.1. Procoagulant activity of fucoidans

Fucoidans' pro- and anticoagulant activities in human plasma were assessed using CAT at a low tissue factor (TF) concentration (Fig. 2). To simulate clotting conditions in hemophilia A, which is characterized by low thrombin generation, we blocked FVIII activity in normal human plasma by adding anti-human-FVIII antibody (Knappe et al., 2013). The thrombin generation profile yields several parameters that describe plasma coagulation. Peak thrombin was selected as primary parameter to depict the concentration-dependent hemostatic effect (Fig. 2A). The resulting bell-shaped concentration profiles are characteristic for fucoidans' *co*-existing pro-and anticoagulant mechanisms (Fig. 2B). All fucoidans corrected impaired thrombin generation of FVIII-inhibited plasma to or above normal levels around 1 μg/mL, while heparin completely eliminated thrombin generation. In contrast, fucoidans' procoagulant activity still outweighed anticoagulant effects occurring at concentrations >10 μg/mL, and did not reach the baseline of FVIII-inhibited plasma (Fig. 2B). Alginate, an impurity of fucoidan extracts, had an effect on thrombin generation in CAT assays at high concentrations. However, given fucoidans' alginate contents (<10–30%; see Table 3), this is not relevant at their optimal procoagulant concentrations. Thus, the fucoidans' procoagulant window spans a wide concentration range (~0.1–100 μg/mL). EC_{50} for procoagulant activity ranged from 0.2 to 0.8 μg/mL (Table 1).

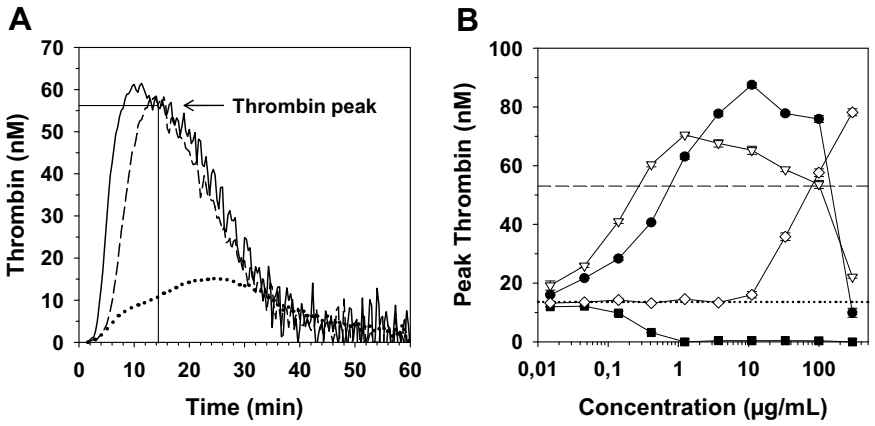

Fig. 2. *Effect of fucoidans, heparin and alginate on thrombin generation in FVIII-inhibited human plasma measured by CAT assay.* (A) Thrombin generation over time in normal (dashed line), FVIII-inhibited plasma (dotted line) and FVIII-inhibited plasma with *F.v.* fucoidan 1 at 0.4 μg/mL (solid line). (B) Concentration-dependent effect of two fucoidans, heparin and alginate on peak thrombin generation: *F.v.* fucoidan (triangles), *E.m.* fucoidan (circles), heparin (squares) and alginate (diamonds). The bell-shaped concentration profiles are characteristic for the co-existing pro- and anticoagulant mechanisms of fucoidans. A wide procoagulant window was observed for fucoidans. Dotted and dashed lines represent thrombin peaks for FVIII-inhibited plasma and normal plasma, respectively. The anticoagulant heparin completely eliminated thrombin generation. Results are shown as averages ± standard errors of duplicate measurements.

3.2. Inhibition of tissue factor pathway inhibitor

Fucoidans exhibited TFPI-neutralizing activity in initial mechanistic studies by binding to C-terminal domains of TFPI (Liu et al., 2006; Zhang et al., 2014). Therefore, the ability to reverse the inhibitory effect of TFPI was also assessed in this study using dilute prothrombin time (dPT) assays. The dPT assay is a modification of the classical plasma clotting assay using reduced TF concentrations as clotting trigger to gain higher sensitivity to TFPI. It primarily detects effects on the extrinsic coagulation pathway which are dependent on TF and tightly regulated by full-length TFPI (fl-TFPI). In this study, normal plasma was supplemented with exogenous fl-TFPI protein to achieve a prolonged clotting time of >150 s. Titration of fucoidan extracts (0.03–1 μg/mL) reversed the effect of TFPI and resulted in decreased clotting times. EC_{50} values for inhibition of TFPI were derived from the sigmoidal dose–response curves. The fucoidans from four species showed similar TFPI-inhibiting activities with EC_{50}s of ~0.4 μg/mL and reversed the TFPI-induced prolonged clotting time to values of normal plasma (~70 s) (Supplemental Fig. 1), confirming that fucoidan procoagulant activity is mediated by TFPI-inhibition.

3.3. Anticoagulant activity

Fucoidans' (0–60 μg/mL) anticoagulant activity was evaluated by aPTT assay, a routine plasma clotting test whereby clotting is initiated by a mixture of contact activators and phospholipids. The test detects changes in the intrinsic pathway of coagulation, but not fucoidans' inhibition of TFPI activity. Unlike CAT, aPTT monitors anticoagulant activities separately from their procoagulant effects. The anticoagulant effect was shown as a concentration-dependent increase in clotting time. Fucoidan concentrations of 4.5–8.7 μg/mL increased clotting time of normal plasma by 50% (Table 1, Supplemental Fig. 2). *U.p.* fucoidan's anticoagulant activity was about twice that of *L.j.*, *F.v.* and *E.m.* fucoidans. All fucoidans displayed increasing anticoagulant activities at concentrations 10–30-fold higher than their EC_{50}s for procoagulant activity.

3.4. Impact on contact activation

Anionic polymers such as sulfated glycosaminoglycans and polyphosphates activate coagulation *via* the intrinsic contact pathway (Melo & Mourao, 2008; Muller et al., 2009; Pan et al., 2010). Systemic activation of this pathway, however, is to be avoided due to initiation of inflammation and anaphylactic reactions (Guerrini et al., 2008; Kishimoto et al., 2008). To rule out fucoidans with similar properties, extracts were tested up to 33 μg/mL in CAT assays with normal human plasma. Adding CTI to plasma inhibits FXIIa and hence blocks the activation of the contact pathway (Dargaud, Luddington, & Baglin, 2006; Mahoney et al., 1984; Ratnoff & Moneme, 1981). Comparing thrombin generation profiles in the absence and presence of CTI revealed the potential for contact activation as shown by a known contact activator OSCS (Supplemental Fig. 3). All four fucoidans improved thrombin generation in normal plasma, indicating a FVIII-independent mode of action. *L.j.* fucoidan showed a marked increase in thrombin generation >5 μg/mL in the absence of CTI compared with the control with CTI. This was not an effect of the contaminant alginate, which did not activate the contact pathway (Supplemental Fig. 3). *E.m.* fucoidan only had a slight effect at concentrations >4 μg/mL; *U.p.* and *F.v.* fucoidan did not stimulate contact activation at all up to 33 μg/mL. This concentration is ~30-fold higher than the optimal procoagulant concentration of about 1 μg/mL (Fig. 2).

Table 1
Pro- and anticoagulant effects of fucoidans: EC_{50} values of procoagulant activity by CAT, and concentration with a 50% increase in clotting time by aPTT. The values are based on duplicate measurements (CV < 5%).

	L.j. fucoidan	*U.p.* fucoidan	*E.m.* fucoidan	*F.v.*		
				Fucoidan 1	Fucoidan 2	Fucoidan 3
CAT EC_{50} (μg/mL)	0.3	0.4	0.8	0.2	0.3	0.2
aPTT (μg/mL)	7.0	4.5	8.7	6.5	6.3	6.2
Ratio aPTT: CAT EC_{50}	23.3	11.3	10.9	32.5	21.0	31.0

L.j. = *Laminaria japonica*, *U.p.* = *Undaria pinnatifida*, *E.m.* = *Ecklonia maxima*, *F.v.* = *Fucus vesiculosus*.

3.5. Structural analysis by gel electrophoresis

We performed extensive analyses to evaluate and compare fucoidans on a structural level. Fucoidans were displayed on agarose gel (Supplemental Fig. 4), which is an inexpensive, simple and effective method to analyze complex sulfated polysaccharides (Volpi & Maccari, 2006). Purity, molecular size-to-charge ratio and affinity to barium affect migration of a sample in the gel. All fucoidans were distinguishable by their migration pattern, and excellent lot-to-lot consistency of *F.v.* fucoidan was confirmed.

3.6. Molecular weight and polydispersity

Fucoidans' *Mw* and polyD were determined by SEC-MALLS (Table 2). The d*n*/d*c* value of *F.v.* fucoidan (0.113 mL/g) was used to calculate the average *Mw* of all fucoidan samples. *F.v.* and *L.j.* fucoidans had an average *Mw* of 130–170 kDa, *U.p.* fucoidan a *Mw* > 500 kDa, and *E.m.* fucoidan a *Mw* > 1000 kDa. PolyD was represented as the weight average molecular weight (*Mw*) divided by the number average molecular weight (*Mn*), or *Mw*/*Mn*. PolyD, which reflects size heterogeneity, was similar for all four fucoidans.

3.7. Monosaccharide analysis using HPAEC-PAD

Before HPAEC-PAD analysis, the fucoidans were hydrolyzed to monosaccharides (Zhang, Khan, Nunez, Chess, & Szabo, 2012), verified by thin layer chromatography (data not shown) (Zhang, Xie, Zhang, & Linhardt, 2007). Representative chromatograms of monosaccharide standards and one fucoidan sample are shown in Supplemental Fig. 5. Monosaccharide composition is listed in Table 2. Fucose, galactose and xylose were the main components in *L.j.* fucoidan, fucose and galactose in *U.p.* and *E.m.* fucoidan, and fucose in *F.v.* fucoidan.

3.8. Fucose, alginate and heterogeneity determinations using ^{13}C NMR

NMR is an effective technique to characterize complex carbohydrates (Chen et al., 2013), and enabled elucidation of the major disaccharide repeat unit in *F.v.* fucoidan (Chevolot, Mulloy, Ratiskol, Foucault, & Colliec-Jouault, 2001). Due to fucoidans' complex structure, quantitative ^{13}C NMR was employed to study fucose and alginate content as well as overall heterogeneity.

Evaluation of relative integrals using various recycle delays showed that relative peak intensities were unchanged after 5 s. This time was also sufficient to remove the nuclear Overhauser enhancement (NOE) that was largest for the methyl carbons. The error in ^{13}C NMR measurements was estimated to be ±10%. Spectra were labelled and integrated over chemical shift (δ) ranges shown in Fig. 3. Carbonyl groups from alginate were observed at δ 170–185 ppm, anomeric peaks at δ 88–112 ppm. Other carbons on the sugar ring were observed at δ 55–88 ppm, the methyl group of fucose at δ 9–20 ppm.

Alginate and fucose contents are listed in Table 3. *L.j.* and *E.m.* fucoidans contained relatively high amounts of alginate, *U.p.* and *F.v.* fucoidans small amounts. Alginate content was also determined by carbazole assay (Supplemental Table 1) and showed a similar trend. NMR results were used for further evaluation as the carbazole assay can be affected by a high content of neutral sugars (Bitter & Muir, 1962). *F.v.* fucoidan was primarily composed of fucose. *L.j.* and *U.p.* fucoidan contained 55–59% fucose, and *E.m.* fucoidan 39%. Fucose contents measured by HPAEC-PAD and NMR were consistent, for the four fucoidans, with slightly higher results for NMR analysis (Tables 2 and 3).

3.9. Elemental analysis

Thirty elements were analyzed using ICP (Table 3, Supplemental Table 2). Sulfur contents varied from 5.8 wt% for *L.j.* to 10 wt% for *U.p.* fucoidan. Sodium, corresponding to the primary counter ion was 6.3 to 7.5 wt%. Only trace amounts of arsenic (As), a toxin of interest for algae sourced fucoidans, were detected, ranging from 0.9 μg/g in *E.m.* to ≤0.1 μg/g in the other fucoidans.

Sulfur contents determined by colorimetric titration and ICP were consistent (Supplemental Tables 2 and 3). Carbon and hydrogen contents were similar for all fucoidans. Nitrogen content was 0.6 wt% in *E.m.*, and ≤0.1% in all other fucoidans (Supplemental Table 3), implying that *E.m.* fucoidan contains more protein.

3.10. Fucoidan ranking

A relative ranking of fucoidan properties is presented in Table 4. All screened fucoidans showed a procoagulant window spanning concentration ranges of four orders of magnitude. However, *L.j.* and *F.v.* fucoidan had slightly higher procoagulant activity than the other extracts. TFPI–dPT assay showed all fucoidans to inhibit TFPI with comparable efficacy (Supplemental Fig. 1) and was therefore not included in the ranking. The ratio of intertwined pro- and anticoagulant activity was most favorable for *F.v.* fucoidan, indicating that the anticoagulant activity is outweighed by the procoagulant effect. *L.j.* fucoidan also had high procoagulant and low anticoagulant activity; while *U.p.* fucoidan had a higher anticoagulant activity. Activation of the contact pathway was mediated by *L.j.* and *E.m.* fucoidans at >5 μg/mL (Supplemental Fig. 3). *U.p.* and *F.v.* fucoidans did not activate the contact pathway within the tested concentration range, which is favorable.

Comparison of structural properties is also critical to select a high quality fucoidan for further study. Generally, larger *Mw* fucoidans are expected to have lower solubility and reduced bioavailability (Tokita, Nakajima, Mochida, Iha, & Nagamine, 2010). Along these lines, high polyD and structural heterogeneity challenge analytical assay development and complicate bioavailability and pharmacokinetics studies, which is not desired.

Impurities in the fucoidan extracts will affect their activities, increase potential toxicity, and impact quality control during processing. Therefore, we analyzed organic impurities as well as

Table 2
Structural properties of fucoidans: molecular weights (*Mw*) and polydispersities (PolyD, *Mw*/*Mn*) determined by SEC-MALLS and monosaccharide composition (% relative area) determined by HPAEC-PAD.

	L.j. fucoidan	*U.p.* fucoidan	*E.m.* fucoidan	*F.v.*		
				Fucoidan 1	Fucoidan 2	Fucoidan 3
Average *Mw* (kDa)	170	620	1360	160	150	130
PolyD	1.8	1.6	1.5	1.6	1.7	1.7
Fucose	39	58	38	73	75	76
Galactose	35	37	41	15	15	14
Glucose	1	3	8	1	0	0
Xylose	21	1	5	9	8	8
Mannose	4	1	8	2	2	2

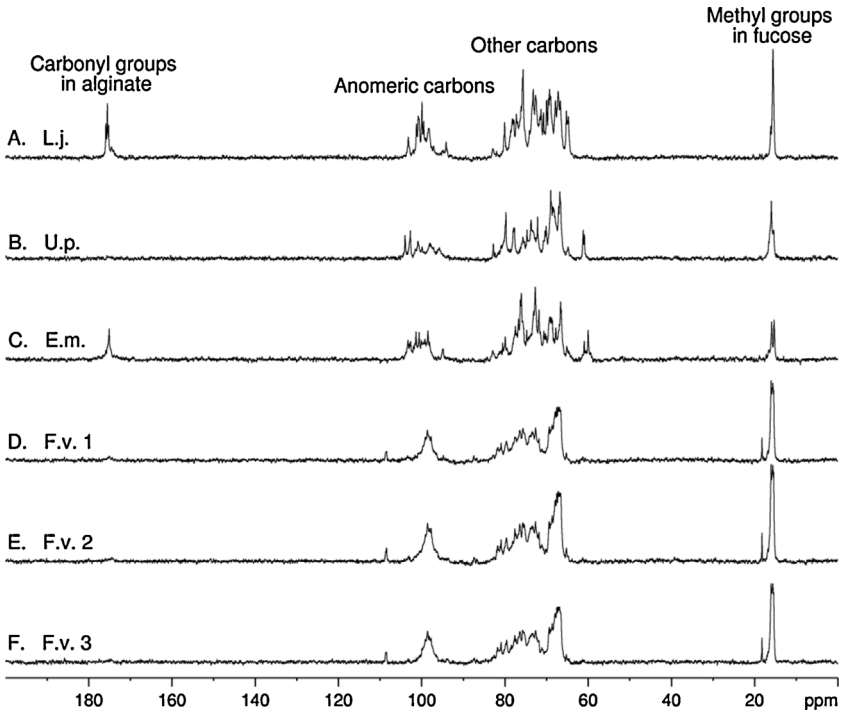

Fig. 3. *Quantitative* ^{13}C *NMR spectra of fucoidans to determine fucose, alginate content and heterogeneity*. Recycle delays of 5 s and an acquisition time of 0.1 s were used for full relaxation of the carbonyl groups and to prevent NOE of all signals in the ^{13}C NMR spectra. Spectra (A)–(C) show fucoidan samples from three algae species, (D)–(F) three lots of a fourth fucoidan species.

inorganic elements. Our results showed inorganic impurities (Supplemental Table 2) to be at low levels. As a major contaminant of fucoidan extracts, alginate was included in the ranking.

Relative ranking of these factors for the six tested fucoidans (Table 4) helps to select a fucoidan with one or more desired properties. Regarding improvement of coagulation, *F.v.* fucoidan was identified as the most suitable for further exploration and optimization.

4. Discussion

Exploratory research begins with selecting several compounds for a subset with the desired biological activities. Fucoidans from different sources have been screened before for biological effects such as anti-inflammatory, anticoagulant, antiangiogenic and anti-adhesive activities (Cumashi et al., 2007; Jin, Zhang, Wang, & Zhang, 2013; Ustyuzhanina et al., 2013). These reports showed that fucoidan activity largely varies with brown algae species and structural characteristics including *Mw* or sulfate content.

We analyzed fucoidans from four brown algae species in detail for structural properties, impurities and effects on coagulation. Finally, fucoidans with optimal activities to potentially promote coagulation in conditions with compromised hemostasis such as hemophilia were identified.

In vitro data demonstrated that fucoidans exert their procoagulant activity by inhibiting TFPI (Liu et al., 2006; Zhang et al., 2014) and by accelerating thrombin-dependent FVa formation (Mutch, Johnson, & Morrissey, 2007). The fucoidans presented here similarly reversed the anticoagulant action of fl-TFPI in a TFPI–dPT assay with low half-maximum effective concentrations (EC_{50}). We therefore conclude that fucoidans primarily stimulate the extrinsic coagulation pathway by inhibiting TFPI. Nevertheless, we also showed that *L.j.* and *E.m.* fucoidan triggered contact activation at concentrations >5 μg/mL, which is in the range of their procoagulant optimum. Contact activation by fucoidans has not been extensively studied. However it is a reason for concern as contact system activation and subsequent generation of bradykinins by OSCS, a contaminant of heparin products, caused adverse

Table 3
Fucoidan impurities: alginate and fucose content based on ^{13}C NMR analysis and elemental analysis of As, Ca, Mg, Na, S, and theoretical S (wt%).

	L.j. fucoidan	*U.p.* fucoidan	*E.m.* fucoidan	*F.v.*		
				Fucoidan 1	Fucoidan 2	Fucoidan 3
Alginate content (%)[a]	28	<10	25	<10	<10	<10
Fucose content (%)	55[b]	59[c]	39[b]	83[c]	86[c]	83[c]
As (wt%)	<0.1	<0.1	0.9	0.1	<0.1	<0.1
Ca wt%	1.8	0.1	0.9	0.2	0.1	0.1
Mg (wt%)	0.1	0.2	0.4	0.2	<0.1	<0.1
Na (wt%)	7.5	7.0	6.9	6.3	6.9	7.2
S (wt%)	5.8	10	6.0	8.7	9.1	9.9
Adjusted S wt% of fucoidan[d]	8.5	10.2	8.4	9.5	9.8	10.5

[a] Calculated using Eq. (1).
[b] Calculated using Eq. (2).
[c] Calculated using Eq. (3).
[d] The theoretical S wt% was calculated from S wt%, alginate content and the monosaccharide composition in Table 2.

Table 4
Relative ranking of critical functional and structural factors for the selection of procoagulant fucoidans species.

Species/lots	*L.j.* fucoidan	*U.p.* fucoidan	*E.m.* fucoidan	*F.v.*		
				Fucoidan 1	Fucoidan 2	Fucoidan 3
Procoagulant activity[a]	(3)	(2)	(1)	(3)	(3)	(3)
Anticoagulant[b]	(2)	(1)	(3)	(2)	(2)	(2)
Ratio[c]	(2)	(1)	(1)	(3)	(2)	(3)
Activation of contact pathway[d]	(1)	(2)	(1)	(2)	(2)	(2)
Mw[e]	(3)	(2)	(1)	(3)	(3)	(3)
Monosaccharide composition[f]	(1)	(3)	(2)	(4)	(4)	(4)
Heterogeneity tested by NMR[g]	(1)	(3)	(2)	(4)	(4)	(4)
Alginate impurity[h]	(1)	(2)	(1)	(2)	(2)	(2)

[a] Procoagulant activities were ranked from (1) to (3), where (1) indicates the highest EC_{50} concentration.
[b] Anticoagulant activities were ranked from (1) to (3), where (1) indicates the lowest concentration to increase clotting time of normal plasma by 50%.
[c] The ratio of anti- to procoagulant activities was ranked from (1) to (3), where (1) indicates the lowest ratio.
[d] Activation of contact pathway was ranked from (1) to (2), where (1) indicates the lowest concentration required to activate the pathway.
[e] *Mw* order ranked from (1) to (3), where (1) indicates the highest *Mw*.
[f] The complexity of the monosaccharide compositions was ranked from (1) to (4), where (1) is the most complex.
[g] Degree of complexity of anomerics and the other carbon region in ^{13}C NMR spectra. The heterogeneity order was ranked from (1) to (4), where (1) is the highest heterogeneity.
[h] Alginate content was ranked from (1) to (2), where (1) is >10% and (2) < 10%.

events in patients (Guerrini et al., 2008; Kishimoto et al., 2008). It remains to be determined which structural features provoke contact activation by fucoidans. In follow-up studies we fractionated and over-/desulfated *F.v.* fucoidan showing that a high degree of sulfation and/or a large *Mw* play a role for FXIIa activation (Zhang et al., 2014). However, *L.j.* fucoidan substantially triggered the contact pathway although it has an intermediate *Mw* and lower sulfate content than the other fucoidans. The major contaminant alginate does not trigger contact activation (Supplemental Fig. 3F). Thus, additional features such as monosaccharide composition, linkages or further extract impurities may be of importance.

The fucoidan's net effect of procoagulant (TFPI inhibition) and anticoagulant activities was determined by a global hemostatic thrombin generation assay. In contrast to the TFPI–dPT assay, the tested fucoidans displayed differences in the CAT assay with EC_{50} values ranging from 0.2 to 0.8 μg/mL. This may be explained by differences in their anticoagulant activity. Fucoidans' anticoagulant activity is the most widely studied biological effect, with potentiation of thrombin inhibitors antithrombin III and/or heparin cofactor II described as relevant mechanisms (Pereira, Mulloy, & Mourao, 1999). Especially the degree of sulfation has been related to this bioactivity (Chen et al., 2013; Haroun-Bouhedja, Ellouali, Sinquin, & Boisson-Vidal, 2000; Pereira, Melo, & Mourao, 2002; Ustyuzhanina et al., 2013). The sulfur content of tested fucoidans varied from 5.8 wt% for *L.j.* to 10 wt% for *U.p.* fucoidan (corresponding to ~19–32 wt% SO_3Na) and were similar to previously published data (Cumashi et al., 2007). The high sulfate content and large *Mw* of *U.p.* fucoidan may explain its higher anticoagulant activity. *F.v.* fucoidan was less anticoagulant despite a sulfur content of about 10 wt%. This may be due to the smaller *Mw* and/or other factors such as sugar composition.

In addition, contaminants in fucoidan extracts can alter sulfur content. The low sulfur contents of *L.j* and *E.m* were partially caused by their high alginate contents, and may not be an indication of the low sulfur contents of fucoidan themselves. For example, the sulfur content of *L.j.* fucoidan was adjusted from 5.8 to 8.5 wt% after excluding alginate (Table 3). In conclusion, sulfur content was not a critical selection factor, but could be considered as a reference in the future.

Other structural properties are also crucial for the selection of a high quality fucoidan extract. Capillary electrophoresis has previously been performed to test heparin-related impurities, dermatan sulfate and contaminant OSCS (Somsen, Tak, Torano, Jongen, & de Jong, 2009; Volpi, Maccari, & Linhardt, 2009; Wielgos, Havel, Ivanova, & Weinberger, 2009). In our studies, we used agarose gel electrophoresis to visualize the high *Mw*, complex fucoidan mixtures. *Mw* and polyD were assessed by SEC-MALLS, monosaccharide composition by HPAEC-PAD, and structural fingerprinting and alginate content by ^{13}C NMR. Anomeric and other regions of the ^{13}C NMR spectra reflected the complexity of, not only fucoidans' sugar compositions, but also their sulfation patterns and linkages. Thus, NMR is a measure of chemical/structural heterogeneity. Meanwhile, polyD is a measure of heterogeneity with respect to size and shape of the components in the mixture and is thus a measure of physical heterogeneity. Both measures of heterogeneity are complementary to one another and are important to characterize the overall heterogeneity of polysaccharides. In general, molecules with high polyD and structural heterogeneity challenge analytical assay development and reduce bioavailability upon subcutaneous or oral administration. The polyD values did not vary over a large range and was, therefore, not identified as a critical selection factor in this particular case. Overall, *F.v.* fucoidan was the least heterogeneous with a high fucose and sulfur content and the lowest *Mw*. Nevertheless, the tested fucoidan extracts were still heterogeneous as seen by agarose gel band patterns. This may necessitate further optimization of fucoidan production for potential therapeutic application.

In addition to molecular characteristics, fucoidan isolation from brown algae may involve co-extraction of other polysaccharides such as alginate and laminaran. Alginate is composed of mannuronic and guluronic acid with 1–4 linkage (Haug, Larsen, & Smidsrod, 1966; Haug, Larsen, Smidsrod, & Painter, 1967). Thus, carbonyl groups from alginate in the ^{13}C NMR spectra were integrated to calculate alginate contents which ranged from <10% to 30% for tested extracts. A wide range of alginate contamination (1–30%) has been reported for fucoidans from different species (Cumashi et al., 2007), while the hexuronic acid content of fucoidan itself is low (Zhang et al., 2012). Thus, alginate determination is important to avoid fucoidan extracts that contain high amounts of such components. Laminaran is composed of glucose with 1–3 and 1–6 linkages (Maeda & Nishizawa, 1968; Nelson & Lewis, 1974). Since the glucose contents of the tested fucoidans were negligible (Table 2), laminaran impurity was not considered a selection criterion.

Other organic impurities, such as acetic acid or glycerol, may be introduced during manufacturing. However, they were not detected in the examined fucoidans by one-dimensional ^{1}H and two-dimensional ^{1}H–^{1}H and ^{1}H–^{13}C NMR, which identify impurities with high specificity (Holzgrabe, Wawer, & Diehl, 1999).

In addition, inorganic metal ions such as As, Cd or Pb are well absorbed by seaweeds and can bind to negatively charged sulfated polysaccharides. Many algae species and algae products have been analyzed for accumulation of toxic cations showing that some of them exceeded tolerable intake limits (Almela, Clemente, Velez, & Montoro, 2006; Besada, Andrade, Schultze, & González, 2009; Sanchez-Rodriguez, Huerta-Diaz, Choumiline, Holguin-Quinones, & Zertuche-Gonzalez, 2001). Therefore, heavy metal content should be examined for natural marine compounds. Only negligible levels of toxic ions were detected in the tested fucoidans with *E.m.* fucoidan having slightly higher levels.

5. Conclusion

In summary, the focus of this study was to screen for fucoidan with high *in vitro* procoagulant activity. Since activity profile and structural properties of fucoidans are complex, numerous factors were considered when evaluating the overall suitability: procoagulant activity, anticoagulant activity, *Mw*, polyD, structural heterogeneity, and impurities. *F.v.* fucoidan was the most adequate for further exploration regarding improvement of coagulation. Beyond that, the presented screening scheme can be applied to other coagulation-relevant and additional activities of fucoidans. The established substance-specific analytical tools will support the identification of preferred properties of this structurally complex class of botanical molecules in the future.

Acknowledgements

We thank Nadia Ivanova, Julia Navik, and Thang Tran for their technical assistance in method validation and sample testing for the structural analysis and Klara Kunckova for performing activity assays. The authors thank Edward Chess and Prasad Dande for their suggestions and comments regarding data interpretation (or analysis) and manuscript writing.

Z. Z. performed fractionation, NMR, HPAEC experiments and wrote the manuscript draft; S. T. performed coagulation analytics and prepared the manuscript; S. K. led activity analysis and reviewed the manuscript; C.Q. and G.J.R. assisted with NMR analysis. J.C. performed SEC-MALLS studies; C.S. led structure analysis; F.S. and M. D. designed the research study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.09.001.

References

Ale, M. T., Mikkelsen, J. D., & Meyer, A. S. (2011). Important determinants for fucoidan bioactivity: A critical review of structure–function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds. *Marine Drugs*, *9*, 2106–2130.

Almela, C., Clemente, M. J., Velez, D., & Montoro, R. (2006). Total arsenic, inorganic arsenic, lead and cadmium contents in edible seaweed sold in Spain. *Food and Chemical Toxicology*, *44*, 1901–1908.

Berteau, O., & Mulloy, B. (2003). Sulfated fucans, fresh perspectives: Structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology*, *13*, 29R–40R.

Besada, V., Andrade, J. M., Schultze, F., & González, J. J. (2009). Heavy metals in edible seaweeds commercialised for human consumption. *Journal of Marine Systems*, *75*, 305–313.

Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, *4*, 330–334.

Capila, I., & Linhardt, R. J. (2002). Heparin–protein interactions. *Angewandte Chemie Internationale Edition in English*, *41*, 391–412.

Chen, S., Li, G., Wu, N., Guo, X., Liao, N., Ye, X., et al. (2013). Sulfation pattern of the fucose branch is important for the anticoagulant and antithrombotic activities of fucosylated chondroitin sulfates. *Biochimica et Biophysica Acta*, *1830*, 3054–3066.

Chevolot, L., Mulloy, B., Ratiskol, J., Foucault, A., & Colliec-Jouault, S. (2001). A disaccharide repeat unit is the major structure in fucoidans from two species of brown algae. *Carbohydrate Research*, *330*, 529–535.

Crawley, J. T., & Lane, D. A. (2008). The haemostatic role of tissue factor pathway inhibitor. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *28*, 233–242.

Cumashi, A., Ushakova, N. A., Preobrazhenskaya, M. E., D'Incecco, A., Piccoli, A., Totani, L., et al. (2007). A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology*, *17*, 541–552.

Dargaud, Y., Luddington, R., & Baglin, T. P. (2006). Elimination of contact factor activation improves measurement of platelet-dependent thrombin generation by calibrated automated thrombography at low-concentration tissue factor. *Journal of Thrombosis and Haemostasis*, *4*, 1160–1161.

Guerrini, M., Beccati, D., Shriver, Z., Naggi, A., Viswanathan, K., Bisio, A., et al. (2008). Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nature Biotechnology*, *26*, 669–675.

Haroun-Bouhedja, F., Ellouali, M., Sinquin, C., & Boisson-Vidal, C. (2000). Relationship between sulfate groups and biological activities of fucans. *Thrombosis Research*, *100*, 453–459.

Haug, A., Larsen, B., & Smidsrod, O. (1966). Study of the constitution of alginic acid by partial acid hydolysis. *Acta Chemica Scandinavica*, *20*, 183–190.

Haug, A., Larsen, B., Smidsrod, O., & Painter, T. (1967). Studies on the sequence of uronic acid residues in alginic acid. *Acta Chemica Scandinavica*, *21*, 691–704.

Hemker, H. C., Giesen, P., al Dieri, R., Regnault, V., de Smedt, E., Wagenvoord, R., et al. (2003). Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiology of Haemostasis and Thrombosis*, *33*, 4–15.

Holzgrabe, U., Wawer, I., & Diehl, B. W. K. (1999). *NMR spectroscopy in drug development and analysis*. Weinheim (Germany): Wiley-VCH Verlag GmbH.

Jin, W., Zhang, Q., Wang, J., & Zhang, W. (2013). A comparative study of the anticoagulant activities of eleven fucoidans. *Carbohydrate Polymers*, *91*, 1–6.

Kishimoto, T. K., Viswanathan, K., Ganguly, T., Elankumaran, S., Smith, S., Pelzer, K., et al. (2008). Contaminated heparin associated with adverse clinical events and activation of the contact system. *New England Journal of Medicine*, *358*, 2457–2467.

Knappe, S., Gorczyca, M. E., Jilma, B., Derhaschnig, U., Hartmann, R., Palige, M., et al. (2013). Plasmatic tissue factor pathway inhibitor is a major determinant of clotting in factor VIII inhibited plasma or blood. *Thrombosis and Haemostasis*, *109*, 450–457.

Lillicrap, D. (2013). The future of hemostasis management. *Pediatric Blood Cancer*, *60*(Suppl 1), S44–S47.

Linhardt, R. J. (1991). Heparin: An important drug enters its seventh decade. *Chemistry and Industry*, *2*, 45–50.

Liu, T., Scallan, C. D., Broze, G. J., Jr., Patarroyo-White, S., Pierce, G. F., & Johnson, K. W. (2006). Improved coagulation in bleeding disorders by non-anticoagulant sulfated polysaccharides (NASP). *Thrombosis and Haemostasis*, *95*, 68–76.

Maeda, M., & Nishizawa, K. (1968). Fine structure of laminaran of *Eisenia bicyclis*. *Journal of Biochemistry*, *63*, 199–206.

Mahoney, W. C., Hermodson, M. A., Jones, B., Powers, D. D., Corfman, R. S., & Reeck, G. R. (1984). Amino acid sequence and secondary structural analysis of the corn inhibitor of trypsin and activated Hageman factor. *Journal of Biological Chemistry*, *259*, 8412–8416.

Melo, F. R., & Mourao, P. A. (2008). An algal sulfated galactan has an unusual dual effect on venous thrombosis due to activation of factor XII and inhibition of the coagulation proteases. *Thrombosis and Haemostasis*, *99*, 531–538.

Morya, V. K., Kim, J., & Kim, E. K. (2012). Algal fucoidan: Structural and size-dependent bioactivities and their perspectives. *Applied Microbiology and Biotechnology*, *93*, 71–82.

Muller, F., Mutch, N. J., Schenk, W. A., Smith, S. A., Esterl, L., Spronk, H. M., et al. (2009). Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell*, *139*, 1143–1156.

Mutch, N. J., Johnson, K. W., & Morrissey, J. H. (2007). Non-anticoagulant sulfated polysaccharides enhance thrombin generation by accelerating factor V activation. *Journal of Thrombosis and Haemostasis*, *5*(Suppl. S2). O-M-094

Nelson, T. E., & Lewis, B. A. (1974). Separation and characterization of the soluble and insoluble components of insoluble laminaran. *Carbohydrate Research*, *33*, 63–74.

Nishino, T., Nishioka, C., Ura, H., & Nagumo, T. (1994). Isolation and partial characterization of a novel amino sugar-containing fucan sulfate from commercial *Fucus vesiculosus* fucoidan. *Carbohydrate Research*, *255*, 213–224.

Pan, J., Qian, Y., Zhou, X., Lu, H., Ramacciotti, E., & Zhang, L. (2010). Chemically oversulfated glycosaminoglycans are potent modulators of contact system activation and different cell signaling pathways. *Journal of Biological Chemistry*, *285*, 22966–22975.

Pereira, M. S., Mulloy, B., & Mourao, P. A. (1999). Structure and anticoagulant activity of sulfated fucans. Comparison between the regular, repetitive, and linear fucans from echinoderms with the more heterogeneous and branched polymers from brown algae. *Journal of Biological Chemistry*, *274*, 7656–7667.

Pereira, M. S., Melo, F. R., & Mourao, P. A. (2002). Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans? *Glycobiology*, *12*, 573–580.

Pomin, V. H., & Mourao, P. A. (2008). Structure, biology, evolution, and medical importance of sulfated fucans and galactans. *Glycobiology*, *18*, 1016–1027.

Prasad, S., Lillicrap, D., Labelle, A., Knappe, S., Keller, T., Burnett, E., et al. (2008). Efficacy and safety of a new-class hemostatic drug candidate, AV513, in dogs with hemophilia A. *Blood*, *111*, 672–679.

Ratnoff, O. D., & Moneme, V. (1981). Inhibition of ellagic acid-activated Hageman factor (factor XII) and Hageman factor fragments by popcorn inhibitor. *Proceedings of the Society for Experimental Biology and Medicine*, *166*, 297–299.

Sanchez-Rodriguez, I., Huerta-Diaz, M. A., Choumiline, E., Holguin-Quinones, O., & Zertuche-Gonzalez, J. A. (2001). Elemental concentrations in different species of seaweeds from Loreto Bay, Baja California Sur, Mexico: Implications for the geochemical control of metals in algal tissue. *Environmental Pollution*, *114*, 145–160.

Skriptsova, A., Shevchenko, N., Zvyagintseva, T., & Imbs, T. (2010). Monthly changes in the content and monosaccharide composition of fucoidan from *Undaria pinnatifida* (Laminariales, Phaeophyta). *Journal of Applied Phycology*, *22*, 79–86.

Somsen, G. W., Tak, Y. H., Torano, J. S., Jongen, P. M., & de Jong, G. J. (2009). Determination of oversulfated chondroitin sulfate and dermatan sulfate impurities in heparin by capillary electrophoresis. *Journal of Chromatography A*, *1216*, 4107–4112.

Tokita, Y., Nakajima, K., Mochida, H., Iha, M., & Nagamine, T. (2010). Development of a fucoidan-specific antibody and measurement of fucoidan in serum and urine by sandwich ELISA. *Bioscience, Biotechnology, and Biochemistry*, *74*, 350–357.

Ustyuzhanina, N. E., Ushakova, N. A., Zyuzina, K. A., Bilan, M. I., Elizarova, A. L., Somonova, O. V., et al. (2013). Influence of fucoidans on hemostatic system. *Marine Drugs*, *11*, 2444–2458.

Vieira, R. P., Mulloy, B., & Mourao, P. A. (1991). Structure of a fucose-branched chondroitin sulfate from sea cucumber. Evidence for the presence of 3-*O*-sulfo-beta-D-glucuronosyl residues. *Journal of Biological Chemistry*, *266*, 13530–13536.

Volpi, N., Maccari, F., & Linhardt, R. J. (2009). Quantitative capillary electrophoresis determination of oversulfated chondroitin sulfate as a contaminant in heparin preparations. *Analytical Biochemistry*, *388*, 140–145.

Volpi, N., & Maccari, F. (2006). Electrophoretic approaches to the analysis of complex polysaccharides. *Journal of Chromatography B: Biomedical Sciences and Applications*, *834*, 1–13.

Wielgos, T., Havel, K., Ivanova, N., & Weinberger, R. (2009). Determination of impurities in heparin by capillary electrophoresis using high molarity phosphate buffers. *Journal of Pharmaceutical and Biomedical Analysis*, *49*, 319–326.

Zhang, Z., Xie, J., Zhang, F., & Linhardt, R. J. (2007). Thin-layer chromatography for the analysis of glycosaminoglycan oligosaccharides. *Analytical Biochemistry*, *371*, 118–120.

Zhang, Z., Khan, N. M., Nunez, K. M., Chess, E. K., & Szabo, C. M. (2012). Complete monosaccharide analysis by high-performance anion-exchange chromatography with pulsed amperometric detection. *Analytical Chemistry*, *84*, 4104–4110.

Zhang, Z., Till, S., Jiang, C., Knappe, S., Reutterer, S., Scheiflinger, F., et al. (2014). Structure-activity relationship of the pro- and anticoagulant effects of *Fucus vesiculosus* fucoidan. *Thrombosis and Haemostasis*, *111*, 429–437.