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Oral Administration of Fucosylated Chondroitin Sulfate Oligomers in Gastro-Resistant Microcapsules Exhibits a Safe Antithrombotic Activity

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Abstract

Fucosylated chondroitin sulfate (FCS) polysaccharide isolated from sea cucumber has potent anticoagulant activity. Based on its resistance to the enzymes present in vertebrates, it may serve as an anticoagulant and shows antithrombotic effects when delivered through gastro-resistant (GR) tablets. However, due to the multiple plasma targets of FCS polysaccharide in the coagulation pathway, bleeding can occur after its oral administration. In the current study, we used FCS oligomers, in particular a mixture of oligosaccharides having 6 to 18 saccharide units, as the active ingredient in GR microcapsules for oral anticoagulation. In a Caco-2 model, the FCS oligomers showed higher absorption than native FCS polysaccharides. Oral administration of FCS oligomer-GR microcapsules provided a dose-dependent, prolonged anticoagulant effect with a selective inhibition of the intrinsic coagulation pathway when compared with *subcutaneous* administration of FCS oligomers or oral administration of unformulated FCS oligomers or native FCS-GR microspheres. Continued oral administration of FCS oligomer-GR microcapsules did not result in the accumulation of oligosaccharides in the plasma. Venous thrombosis animal models demonstrated that FCS oligomers delivered via GR microcapsules produced a potent antithrombotic effect dependent on their anticoagulant properties in the plasma, while oral administration of unformulated FCS oligomers at the same dose exhibited a weaker antithrombotic effect than the formulated version. Oral administration of FCS oligomer-GR microcapsules resulted in no bleeding, while oral administration of native FCS-GR microcapsules resulted in bleeding ($p < 0.05$). Our present results suggest that a FCS oligomer-GR microcapsule formulation represents an effective and safe oral anticoagulant for potential clinical applications.

Keywords
- fucosylated chondroitin sulfate oligomers
- gastro-resistant microcapsules
- oral administration
- anticoagulant and antithrombotic activities
- bleeding tendency

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Introduction

Thrombotic diseases are a serious human health concern and have been a leading cause of death throughout the world over the past few decades.[1,2] Unfractionated heparin and low-molecular-weight heparins have been in widespread and long-term use as anticoagulants.[3–5] However, these glycosaminoglycans have limited bioavailability as they only show anticoagulant and antithrombotic activities following *intravenous* or *subcutaneous* parenteral administration[6] and exhibit serious bleeding risks.[7–9] Newly developed oral anticoagulants, such as dabigatran, rivaroxaban, and apixaban, have predictable pharmacokinetics with selective inhibition on human factor Xa (FXa) or thrombin (factor IIa [FIIa]); however, these agents still show potential bleeding risks in clinical applications.[10–13]

Fucosylated chondroitin sulfate (FCS), extracted from sea cucumber, is a potent anticoagulant polysaccharide attributed to the presence of unique sulfated fucose branches.[14,15] This sulfated polysaccharide, prepared from a marine organism, is resistant to enzymes in the vertebrates' gastrointestinal (GI) tract due to its distinct chemical structure.[14] However, very high doses of FCS polysaccharides are required to show the pharmacological effects under oral administration because of the partial hydrolysis of sulfated fucose branches in gastric acid and its low absorption in the intestinal tract due to its relatively high molecular weight (M_w).[16] Gastro-resistant (GR) tablets prepared with FCS polysaccharides were made to overcome the destruction of FCS by acidic gastric liquid and these exhibit improved anticoagulant effects and inhibit venous thrombosis.[17] However, a bleeding tendency was observed for FCS polysaccharides because in plasma they act on multiple targets in the coagulation pathway.[18]

FCS oligosaccharides can be prepared in several ways from FCS polysaccharides and retain potent anticoagulant and antithrombotic activities without the in vitro and in vivo side effects associated with FCS polysaccharides.[19–22] Recently, we established a method for the rapid preparation of purified FCS oligosaccharides, specifically 6 to 18 oligomers, from the dry sea cucumber, *Pearsonothuria graeffei*. The resulting FCS oligomers exhibit potent anticoagulant and antithrombotic activities by selectively inhibiting the intrinsic factor Xase complex (FXase, factor IXa–VIIIa–Ca^{2+}–phospholipid complex) in the coagulation pathway, while avoiding side effects such as bleeding and hypotension in vivo.[23] FCS oligomers have a potential advantage over the FCS polysaccharide: a significantly lower M_w allowing their intestinal absorption.[24] Thus, we examined the possibility of using FCS oligomers for oral anticoagulation.

Chitosan-coated alginate microcapsules are easily prepared and have been successfully used to orally deliver a wide range of encapsulated drugs, peptides, and proteins to the intestinal tract.[25–28] In the present study, we prepared FCS oligomer-GR microcapsules using a chitosan-coated alginate system. The FCS oligomer-GR microcapsules were orally administrated to rats to evaluate their in vivo anticoagulant and antithrombotic activity and bleeding side effect. We also compared the efficacy of *subcutaneously* administered FCS oligomers, orally administered unformulated FCS oligomers, and oral administered native FCS-GR microcapsules.

Methods

Native FCS polysaccharides were extracted from sea cucumber *P. graeffei* and the FCS oligomers were prepared through a controlled depolymerization of FCS polysaccharides. Detailed information about the preparation process and products were provided in previous publications.[22,23] In addition, a brief summary of the FCS oligomers' main characteristics, including their structure, the proportion of each fraction, the average M_w, and the degree of sulfation, is presented in ►**Supplementary** ►**Fig. S1** and ►**Table S1** (available in the online version). All other chemicals and reagents used were of analytical grade. Preparation of the FCS oligomer-GR microcapsules as well as process optimization is shown in the Supporting Information.

Scanning Electron Microscopy Analysis of the GR Microcapsules

The surface morphology and internal structure of the GR microcapsules were examined using a Hitachi TM1000 tabletop scanning electron microscope (SEM; Ramsey, New Jersey, United States) as described previously.[29] The lyophilized samples were mounted on aluminum stubs, gold coated, and then examined under vacuum. After observing the whole microcapsule a partial longitudinal cut was performed to observe their cross-section.

In Vitro Release of FCS Oligomers from the GR Microcapsules

The lyophilized samples were added into simulated gastric fluid (10 mg/mL pepsin, pH 1.2) and simulated intestinal fluid (10 mg/mL trypsin, pH 6.8), successively. Both the simulated fluids were stirred with 60 rpm at 37°C and sampled every 20 minutes to calculate the release rate of FCS oligomers according to the following formula:

$$A\% = \frac{a_2}{a_1} \times 100\%$$

where $A\%$: the release rate of FCS oligomers;

a_1: the quantity of FCS oligomers in added microcapsules (mg);

a_2: the quantity of released FCS oligomers in the simulated fluid (mg), calculated based on the FCS oligomer standard curve (►**Supplementary Fig. S2**, available in the online version).

Simulated Intestinal Absorption of Native FCS and FCS Oligomers Using a Caco-2 Model

The Caco-2 cells were cultured according to a standard procedure.[30] After reaching confluence, the cells were digested by trypsin and then inoculated on the villi surface of a 6-well transwell (►**Supplementary Fig. S4**, available in the online version) while controlling the density at 10^5 cells/well. A 3 mL

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aliquot of Dulbecco's modified eagle medium (DMEM) was added to both chambers, and the cells were cultured at 37°C in a 5% CO_2 atmosphere. The DMEM was changed every day, until the transepithelial electrical resistance of the Caco-2 cell layer was over 500 Ω/cm^2, which was sufficient for the transwell test.

A 10 mg/mL sample of native FCS or FCS oligomers was prepared with Hank's balanced salt solution (HBSS; pH 7.4). After rinsing the enterocyte, 1.5 mL of native FCS or FCS oligomers and HBSS solution was added to the upper chamber and 2.6 mL of HBSS blank solution was added to the lower chamber. The transwell was incubated at 37°C in a 5% CO_2 atmosphere for 2 hours. The recovered native FCS and FCS oligomers from the transwell were analyzed by high-efficiency gel permeation chromatography (HPGPC) and ^{1}H NMR spectroscopy.

The recovered native FCS and FCS oligomers from the upper and lower chambers were desalted and then made into a same volume of aqueous solution for HPGPC injection. HPGPC of native FCS was profiled on two analytical columns: TSK G4000SWXL 7.8 mm × 30 cm, 8 µm in series with TSK G3000SWXL 7.8 mm × 30 cm, 5 µm (Tosoh Corporation, Tokyo, Japan). Elution was performed with 0.1 M CH_3COONH_4 with 0.02% (*w*/*v*) NaN_3 at a flow rate of 0.6 mL/min, and was monitored with a refractive index (RI) detector. HPGPC of FCS oligomers was profiled on a column (10 × 300 mm) of Superdex Peptide 10/300 GL (GE Healthcare, Chicago, Illinois, United States). Elution was performed with 0.2 M NH_4HCO_3 at a flow rate of 0.4 mL/min, and was monitored with a RI detector. The absorption of the native FCS or FCS oligomers was calculated according to the following formula:

$$B\% = \frac{b_2}{b_1 + b_2} \times 100\%$$

where $B\%$: the absorption of the native FCS or FCS oligomers;
b_1: the HPGPC profile area of the lower-chamber-recovered one;
b_2: the HPGPC profile area of the upper-chamber-recovered one.

For NMR spectroscopy, samples were dissolved in 500 µL of D_2O (99.9%) and lyophilized three times to substitute the exchangeable protons with deuterium, and then transferred to NMR microtubes after dissolving in 500 µL D_2O. The ^{1}H NMR spectrum was recorded on Bruker 600 spectrometer (Madison, Wisconsin, United States) with topspin 3.2 software at 298.15 K.

Administration of Anticoagulants in Rats

Male Sprague Dawley rats (~250 g body weight) were randomly segregated into various groups of five animals each. The "*subcutaneous* administration of 10 mg/kg FCS oligomers" group was administered with100 µL of 0.86% NaCl aqueous solution including 2.5 mg FCS oligomers for each animal. The control group was orally administered with 500 µL distilled water by gavage for each animal. The "oral administration of 50 mg/kg FCS oligomers in aqueous solution" group was administered with 500 µL distilled water including 12.5 mg FCS oligomers by gavage for each animal. The "oral administration of 10 mg/kg rivaroxaban" group was administered with 500 µL distilled water including 2.5 mg rivaroxaban (Bayer Medical, Germany) by gavage for each animal. The groups of oral administration of FCS oligomer-GR microcapsules and native FCS-GR microcapsules were given doses based on the FCS oligomers or native FCS contained in the microcapsules. The preparation of native FCS-GR microcapsules and their simulated release are described in the Supporting Information. The administration of the GR microcapsules was performed as described in a previous publication,[17] the rats were lightly anesthetized with isoflurane, and the quantified and squeezed microcapsules were placed gently near the glottis of the animal, which swallowed them by reflex mechanisms. Institutional guidelines for animal care and experimentation and all tests including the following ones were approved by the Animal Care Review Committee, Zhejiang University.

Anticoagulant Properties and Coagulation Factor Activities in Plasma

Tail vein blood samples (nine volumes) from various groups at different intervals after the administration were collected and immediately mixed with 3.8% sodium citrate aqueous solution (one volume). The anticoagulant properties of the plasma samples, including activated partial thromboplastin time (APTT) and thrombin time (TT), were determined with a coagulometer using the APTT and TT reagents (Teco Medical, Germany) according to the manufacturer's specifications. The anticoagulant properties were expressed as T_1/T_0, which is the ratio between the clotting time in the presence and absence of anticoagulants in the sample. The same plasma samples were incubated in 96-well plates for the assessment of coagulation factor activities including the FXa and FIIa. The plasma samples were incubated for 1 minute at 37°C with Tris-HCl/PEG buffer (0.02M Tris-HCl, 0.15 M NaCl, and 1.0 - mg/mL polyethylene glycol 8000 Da, pH 7.4), and then FXa chromogenic substrate SXa-11(Hyphen Biomed, France) or FIIa chromogenic substrate CS-31(02) (Hyphen Biomed, France) was added to initiate the reaction. The absorbance of the reaction mixture at 405 nm was recorded for 5 minutes. The rate of change of absorbance was proportional to the residual FXa or FIIa activity in the well.

Venous Thrombosis

Antithrombotic activity in rats was assessed using rabbit brain thromboplastin as the thrombogenic stimulus.[31] After the administration of anticoagulants, the rats were anesthetized with an intramuscular injection of 100 mg/kg body weight of ketamine and 16 mg/kg body weight of xylazine, the inferior vena cava and its branches were isolated, and the branch of inferior vena cava under the left renal vein was ligated. A volume of 1 mL/kg body weight of 2% tissue thromboplastin was injected from the femoral vein. After 20 seconds, stasis was established by ligating the edge of the

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left renal vein. After a 20-minute stasis, the cavity was then reopened, the ligated segment was opened longitudinally, and the thrombus formed was removed, rinsed, dried for 24 hours at 50°C, and then weighed.

Bleeding Effects

Blood loss was determined by measuring the hemoglobin present in the water using a spectrophotometric method.[31] At the preset time after the administration of anticoagulants, the rats were anesthetized with a combination of xylazine and ketamine as described earlier. Then the tails of the rats were cut 5 mm from the tip and immersed in 40 mL of distilled water for 60 minutes at 37°C with stirring. The volume of blood was determined from a standard curve based on absorbance at 540 nm.

Statistical Analysis

The data were analyzed using one-way analysis of variance followed by Duncan's multiple-range test using IBMSPSS statistics. Experimental results were reported as the means ± standard deviations. p-Values less than 0.05, 0.01, or 0.001 were considered to be statistically significant (i.e., $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$).

Results

FCS Oligomers are Evenly Distributed in the GR Microcapsules

We compared the SEM characterization of the blank-GR microcapsule and FCS oligomer-GR microcapsule after lyophilization (►Fig. 1). Both of the lyophilized GR microcapsules were crumpled into spheres of ~1 mm particle size. The surface of the blank-GR microcapsule was smooth, while the surface of the FCS oligomer-GR microcapsule was rough as though full of something like villi. We speculate that the hydrogen bond between the FCS oligomer and sodium alginate affects the electrostatic interaction between chitosan and calcium alginate, so that the chitosan crystallized and appeared to be villi during the lyophilization.[32] Meanwhile, the cross-section of the FCS oligomer-GR microcapsule showed more abundant layered structures inside than that of the blank-GR microcapsule. This experiment indicated that FCS oligomers are evenly distributed within the GR microcapsule.

FCS Oligomers from the GR Microcapsules are Selectively Released in a Simulated Intestinal Environment

We simulated the release of FCS oligomers from the GR microcapsules in the digestive tract in vitro (►Fig. 2). In simulated gastric digestion (0–120 minutes), the release rate of FCS oligomers was at a very low level (<10%), indicating that the capsule wall effectively prevents the core content from releasing into the gastric environment. In simulated intestinal absorption (120–420 minutes), the release rate of FCS oligomers reached a high level (~70%) within the first 30 minutes, indicating that the capsule wall collapsed quickly in the intestinal environment releasing FCS oligomers from the capsule core. Some FCS oligomers, bound to the capsule wall, released slowly as the capsule wall gradually degraded in the intestinal fluid. The final release of FCS oligomers reached approximately 82%.Thus, we suggest that FCS oligomer-GR microcapsules could also selectively release the FCS oligomers in the intestinal tract in vivo.

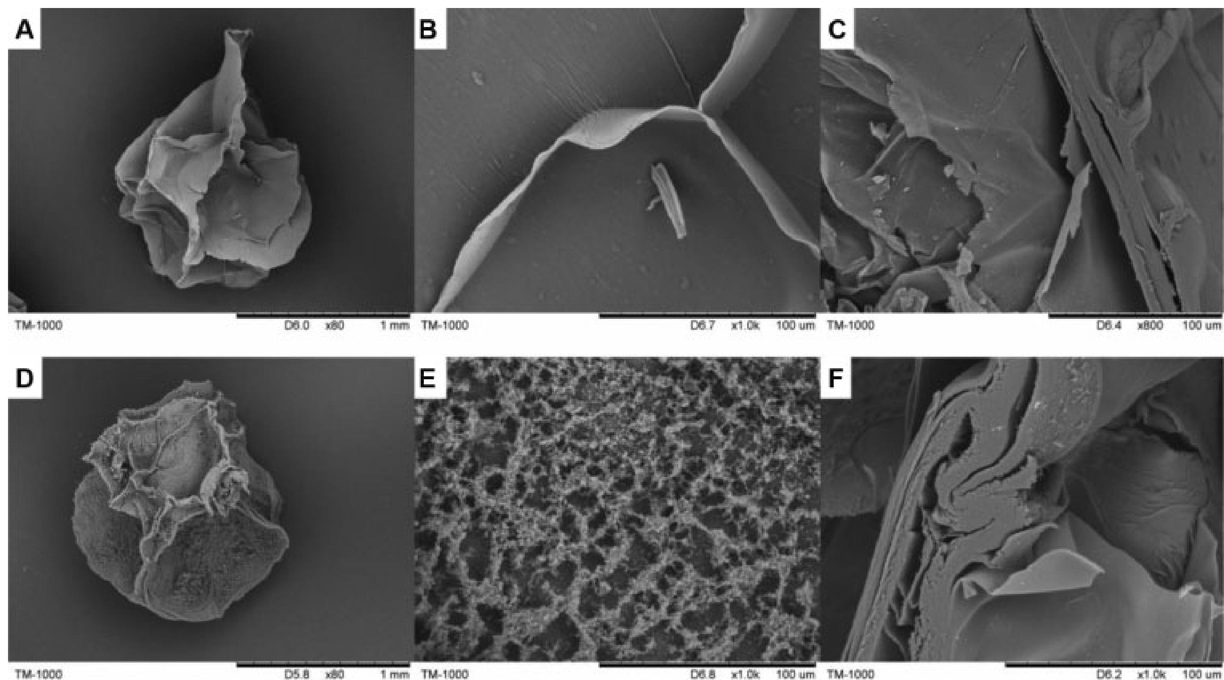

Fig. 1 SEM characterization of the lyophilized GR microcapsules. (**A**) Full view of a blank-GR microcapsule, (**B**) surface of a blank-GR microcapsule, (**C**) cross-section of a blank-GR microcapsule, (**D**) full view of a FCS oligomer-GR microcapsule, (**E**) surface of a FCS oligomer-GR microcapsule, and (**F**) cross-section of a FCS oligomer-GR microcapsule. FCS, fucosylated chondroitin sulfate; GR, gastro-resistant; SEM, scanning electron microscopy.

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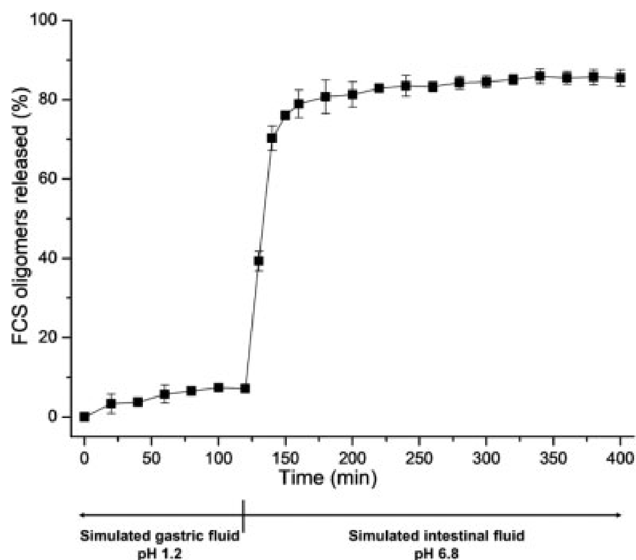

Fig. 2 Simulated release of the FCS oligomers from the GR microcapsules in the GI tract. The time of 0–120 minutes was the gastric stage with a simulated fluid (10 mg/mL pepsin, pH 1.2). Successively, 120–420 minutes was the intestinal stage with a simulated fluid (10 mg/mL trypsin, pH 6.8). Both the simulated fluids were stirred with 60 rpm at 37°C and sampled every 20 minutes to calculate the release rate of FCS oligomers. FCS, fucosylated chondroitin sulfate; GI, gastrointestinal; GR, gastro-resistant.

FCS Oligomers Exhibit Higher Absorption Efficiency than Native FCS in a Caco-2 Cell System

Furthermore, we simulated the absorption efficiency of the native FCS and FCS oligomers in the intestinal tract by using a Caco-2 model. It showed that both the native FCS and FCS oligomers could cross the monolayer intestinal cell, since we observed the FCS polysaccharides or oligosaccharides in the lower chamber as reflected by the HPGPC profiles and NMR spectra (►**Fig. 3**). The absorption efficiency of FCS oligomers was approximately 32%. This was significantly higher than that of native FCS, which was only approximately 9%. However, the M_w distribution of the FCS oligomers did not obviously change after absorption. It indicated that the molecular sizes of the 6 to 18 oligomers show no significant effect on the absorption efficiency for FCS oligosaccharides. But the FCS polysaccharides are much less absorbed by a Caco-2 cell system than the oligosaccharides. By comparing the ^{1}H NMR spectra of the native FCS and FCS oligomers before and after absorption (►**Fig. 3C**), we found that the sulfated fucose branches were stable during the Caco-2 cells' transfer. The sulfated fucose branches are important for the anticoagulant and antithrombotic properties of FCS polysaccharides and oligosaccharides in the plasma level.[33]

Oral Administration of FCS Oligomer-GR Microcapsules Exhibits Intrinsic Anticoagulant Effect In Vivo

In the in vitro studies, we confirmed that the FCS oligomer-GR microcapsules could selectively release most of the FCS oligomers in the simulated intestinal tract, and a substantial amount of the FCS oligomers could be absorbed by a Caco-2 cell system. We now investigate whether the FCS oligomer-GR microcapsules could produce anticoagulant and antithrombotic effects in vivo.

To clarify this question, FCS oligomer-GR microcapsules were orally administered to rats at a dose of 10 or 50 mg/kg. As a comparison, FCS oligomers were also *subcutaneously* administered to rats at a dose of 10 mg/kg. Plasma samples were collected before and every hour after administration (a total of six times) to test the anticoagulant properties and coagulation factor activities. At 1 hour after the *subcutaneous* administration of 10 mg/kg FCS oligomers, an approximately fourfold increase in APTT was observed. Then the APTT rapidly decreased to the initial value at 5 hours after the administration (►**Fig. 4A**). In addition, at 2 hours after the oral administration of 50 mg/kg FCS oligomer-GR microcapsules, we also observed an approximately fourfold increase in APTT as the anticoagulant peak. Then the APTT gradually decreased to its initial value in another 3 hours. The broader APTT curve indicates that oral administration of FCS oligomer-GR microcapsules could provide a longer time of anticoagulant effects in vivo than *subcutaneous* administration of FCS oligomers. However, a fivefold higher dose of FCS oligomers was required by oral administration of GR microcapsules to reach a similar increase in APTT at the anticoagulant peak when compared with *subcutaneous* administration. Oral administration of 10 mg/kg FCS oligomer-GR microcapsules elicited a similar trend in its APTT curve but with much weaker intensity than oral administration of 50 mg/kg FCS oligomer-GR microcapsules. We also monitored the TT after above three administrations (►**Fig. 4B**). However, there was no obvious increase in the TT during the entire process. These results are in accord with a previous in vitro study that showed that FCS oligomers could prolong the APTT but not the TT, due to its selective inhibition on intrinsic FXase.[23]

For further comparison, FCS oligomers in aqueous solution and native FCS-GR microcapsules were also orally administrated to rats at a dose of 50 mg/kg with monitoring the anticoagulant properties and coagulation factor activities in a same way. Oral administration of 50 mg/kg native FCS-GR microcapsules exhibited a similar trend in the APTT curve but with a weaker intensity than oral administration of 50 mg/kg FCS oligomer-GR microcapsules (►**Fig. 4C**). This might be caused by the poor absorption of the native FCS in the intestinal tract, even though the native FCS exhibited a much greater prolongation of APTT than the FCS oligomers in vitro.[23] Oral administration of 50 mg/kg FCS oligomers in aqueous solution showed a weaker prolongation of APTT at every time point and a faster decrease of APTT after the anticoagulant peak than oral administration of 50 mg/kg FCS oligomer-GR microcapsules. It indicated that the GR microcapsules could protect FCS oligomers against the partial removal of sulfated fucose branches in the gastric fluid.[17] Oral administration of 50 mg/kg native FCS-GR microcapsules could also prolong the TT with a similar intensity as APTT (►**Fig. 4D**). It was in accordance with the previous result that native FCS could prolong TT in vitro.[23,34]

We also tested the residual activities of FXa and FIIa for each plasma sample as shown in ►**Fig. 5**. All the residual FXa activity curves exhibited a high correlation with the corresponding

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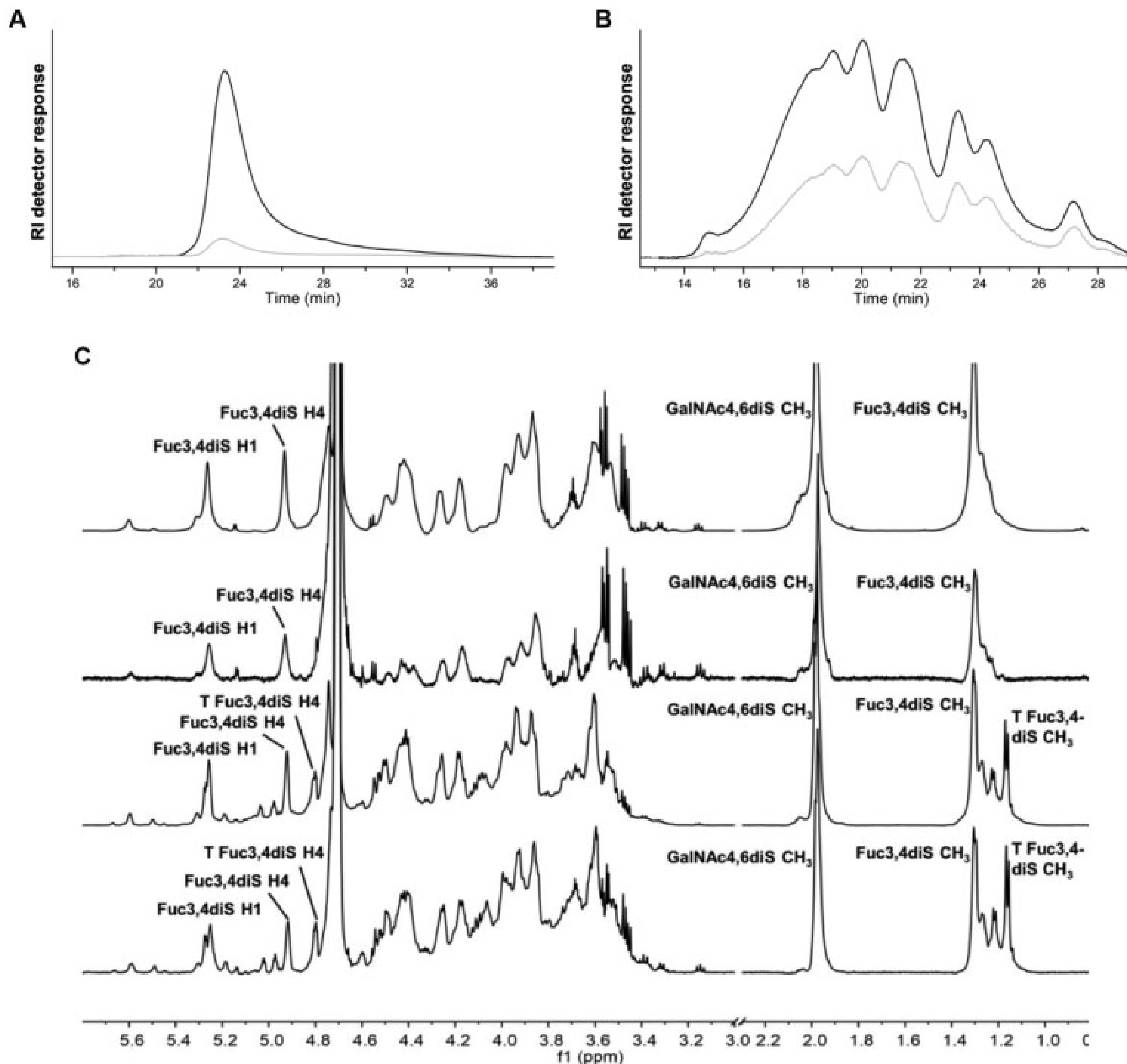

Fig. 3 (**A**) HPGPC profiles of the recovered native FCS from the Caco-2 model. (**B**) HPGPC profiles of the recovered FCS oligomers from the Caco-2 model, the *black line* corresponds to the recovered sample from the upper chamber and the *gray line* corresponds to the recovered sample from the lower chamber. (**C**) ^{1}H NMR spectra of the recovered samples from the Caco-2 model. Spectra from top to bottom correspond to the recovered native FCS from the upper chamber, the recovered native FCS from the lower chamber, the recovered FCS oligomers from the upper chamber, and the recovered FCS oligomers from the lower chamber, respectively. Labels "Fuc3,4diS," "T Fuc3,4diS," and "GalNAc4,6diS" represent the middle fucose residue with 3,4-disulfation, the terminal fucose residue with 3,4-disulfation, and the *N*-acetyl-galactosamine residue with 4,6-disulfation, respectively. The assignments of chemical shifts in the spectra for key structure were accorded to previous publications.[22,23] FCS, fucosylated chondroitin sulfate; GR, gastro-resistant; HPGPC, high-efficiency gel permeation chromatography.

APTT curves. For instance, the lowest residual FXa activity time point corresponded to the highest APTT time point. The tendency of residual FXa activity to fall and rise strictly corresponded to the tendency of APTT to rise and fall. This demonstrates that the prolonging of APTT was closely related to low FXa activity. The residual FIIa activity curves also exhibited high correspondence to the TT curves. Of all the administrations, only the oral administration of 50 mg/kg native FCS-GR microcapsules could reduce the FIIa activity as well as prolong the TT. The other administrations exhibited little influence on the FIIa activity and the TT. This also demonstrates that FCS oligomers delivered by oral administration could also selectively inhibit the intrinsic FXase, but that native FCS delivered by oral administration exhibit multiple anticoagulant mechanisms.

FCS Oligomers Orally Administrated by GR Microcapsules Would Not Accumulate in Plasma

We also performed additional experiments to evaluate the residual level of FCS oligomers or native FCS in plasma after continuous delivery by GR microcapsules. FCS oligomer-GR microcapsules or native FCS-GR microcapsules were orally administrated to rats at a dose of 50 mg/kg every 12 hours for 5 days. Plasma samples were collected 5 hours after the first administration, on the first, third, and fifth days. The APTT and TT as well as FXa and FIIa activities of the plasma samples

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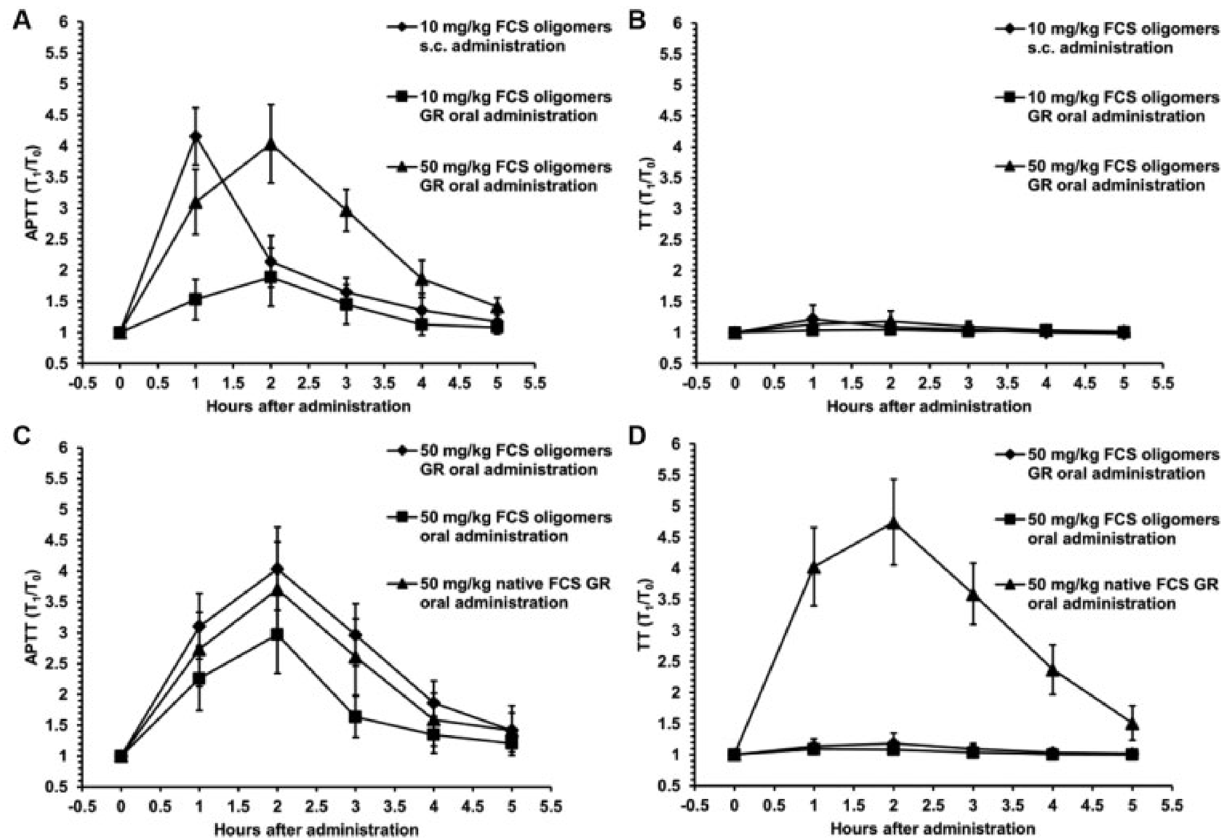

Fig. 4 Anticoagulant properties including APTT (**A** and **C**) and TT (**B** and **D**) after oral administration of FCS oligomer-GR microcapsules, FCS oligomers in aqueous solution, native FCS-GR microcapsules, and *subcutaneous* (s.c.) administration of FCS oligomers. Tail vein blood samples (nine volumes) from various groups at different intervals after the administration were collected and immediately mixed with 3.8% sodium citrate aqueous solution (one volume) for measuring the APTT and TT. Results were expressed as T_1/T_0, which is the rate of APTT (or TT) in the presence or absence of anticoagulants (mean ± SD, $n = 5$). APTT, activated partial thromboplastin time; FCS, fucosylated chondroitin sulfate; GR, gastro-resistant; SD, standard deviation; TT, thrombin time.

were measured. We could see that oral administration of 50 mg/kg FCS oligomer-GR microcapsules did not obviously prolong the APTT or TT, or reduce the FXa or FIIa activity on the third or fifth day (►**Fig. 6**). However, oral administration of 50 mg/kg native FCS-GR microcapsules gradually prolonged the APTT and TT on the third and fifth days. Residual FXa and FIIa activities in the plasma also gradually decreased on the third and fifth days (►**Fig. 6**). This indicates that the continuous oral administration of FCS oligomer-GR microcapsules never resulted in the accumulation of FCS oligomers in the plasma. However, continuous oral administration of native FCS-GR microcapsules caused the accumulation of native FCS polysaccharides in the plasma as reflected by the anticoagulant properties and coagulation factors activities.

Oral Administration of FCS Oligomer-GR Microcapsules Exhibits Potent Antithrombotic Effect In Vivo

We first investigated the antithrombotic effect at 2 and 5 hours after oral administration of 50 mg/kg FCS oligomer-GR microcapsules (►**Fig. 7A**). Antithrombotic activity was investigated in male Sprague Dawley rats with the rabbit brain thromboplastin-induced venous thrombosis model.[31] This showed an antithrombotic effect of approximately 82% venous thrombosis inhibition at 2 hours after oral administration. However, at 5 hours after the oral administration, no antithrombotic effect was observed. This suggested that the antithrombotic effect was derived from the anticoagulant properties of the FCS oligomers in the plasma.

Subsequently, we compared the antithrombotic effects at 2 hours after oral administration of 10 or 50 mg/kg FCS oligomer-GR microcapsules, 50 mg/kg FCS oligomers in aqueous solution, 50 mg/kg native FCS-GR microcapsules, and 10 mg/kg rivaroxaban. Oral administration of 10 mg/kg FCS oligomer-GR microcapsules exhibited a weak antithrombotic effect of approximate 19% venous thrombosis inhibition, due to a low content of FCS oligomers in the plasma (►**Fig. 7B**). Oral administration of 50 mg/kg FCS oligomers delivered by aqueous solution exhibited a weaker antithrombotic effect than observed with GR microcapsules, probably due to the partial removal of sulfated fucose branches in the acid gastric fluid. Oral administration of 50 mg/kg FCS oligomer-GR microcapsules exhibited similarly potent antithrombotic effect as oral administration of 50 mg/kg native FCS-GR microcapsules or 10 mg/kg rivaroxaban.

Oral Administration of FCS Oligomer-GR Microcapsules Exhibits No Bleeding Risk In Vivo

A blood loss mode[31] was performed at 2 hours after the oral administration of 50 or 100 mg/kg FCS oligomer-GR microcapsules, 50 mg/kg native FCS-GR microcapsules, or

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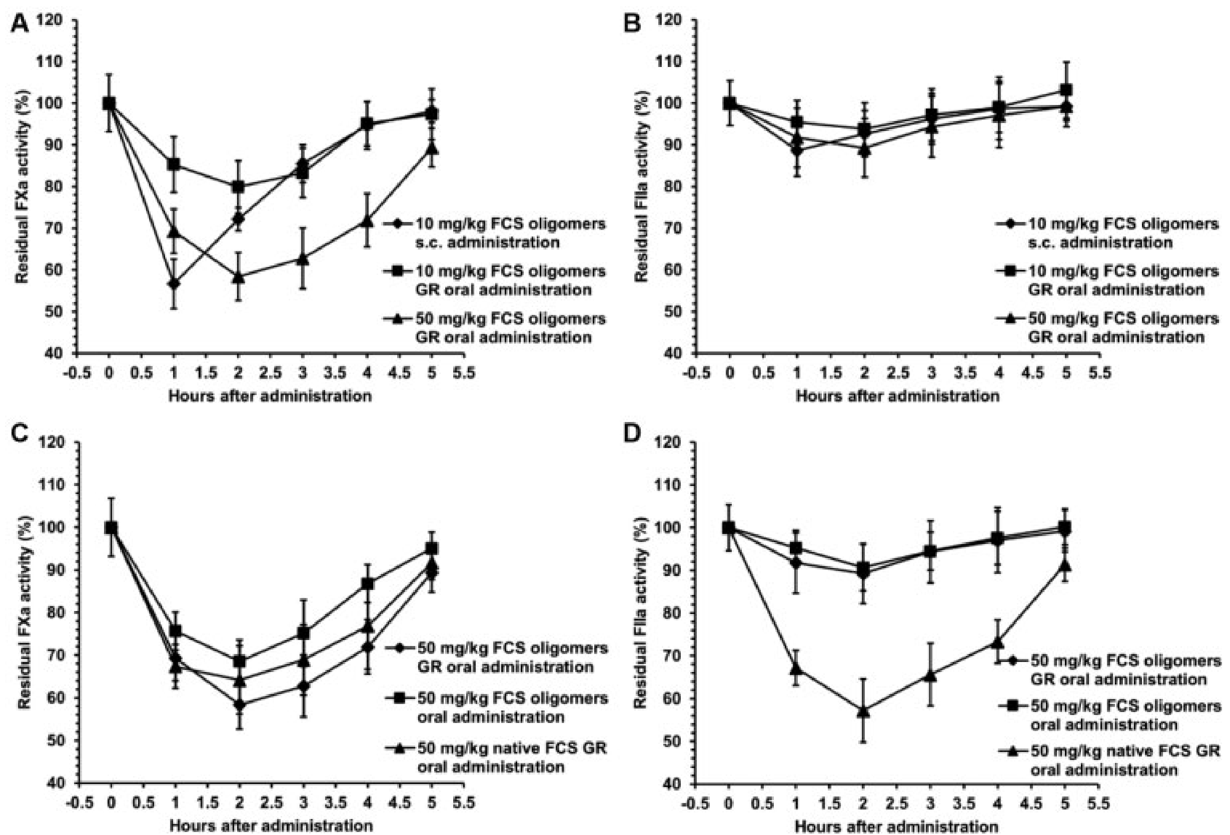

Fig. 5 Residual activities of coagulation factors including FXa (**A** and **C**) and FIIa (**B** and **D**) after oral administration of FCS oligomer-GR microcapsules, FCS oligomers in aqueous solution, native FCS-GR microcapsules, and *subcutaneous* (s.c.) administration of FCS oligomers. Tail vein blood samples (nine volumes) from various groups at different intervals after the administration were collected and immediately mixed with 3.8% sodium citrate aqueous solution (one volume) for measuring residual activities of FXa and FIIa. Results were expressed as the rate of FXa (or FIIa) activity in the presence or absence of anticoagulants (mean ± SD, $n = 5$). FIIa, factor IIa; FXa, factor Xa; FCS, fucosylated chondroitin sulfate; GR, gastro-resistant; SD, standard deviation.

10 mg/kg rivaroxaban to evaluate bleeding risk. Oral administration of 10 mg/kg rivaroxaban clearly exhibited a bleeding tendency ($p < 0.01$), which was in accord with a previous report[17] (►**Fig. 8**). Oral administration of 50 mg/kg native FCS-GR microcapsules also exhibited a bleeding tendency ($p < 0.05$). However, oral administration of 50 or 100 mg/kg FCS oligomer-GR microcapsules exhibited no bleeding risk. This demonstrated that the FCS oligomers in plasma delivered by GR microcapsules selectively inhibit the intrinsic FXase so as to avoid the bleeding risk.

Discussion

The structure of the FCS oligomer-GR microcapsule and its release process in the GI tract are shown in ►**Supplementary Fig. S5** (available in the online version) in a simplified model based on previous publications.[35,36] A mixture of FCS oligomers and sodium alginate was in the core of the microcapsule, which was encased with a cover of calcium alginate. A thin layer of chitosan was used to reinforce the microcapsule through the electrostatic interaction of chitosan with calcium alginate. Once the microcapsule arrives in the stomach, the chitosan layer is dissolved in gastric acid, but the calcium alginate layer remains intact in the low pH environment. The calcium alginate-covered microcapsule then moves into the intestinal tract where the calcium alginate begins to dissolve in the neutral pH environment and suddenly releases all the FCS oligomers present in the core. However, the release of FCS oligomers did not reach 100%. This is probably because a portion of the FCS oligomers, close to the microcapsule wall, is blocked by residual chitosan or calcium alginate.

The absorption of FCS polysaccharides or oligosaccharides is most likely through endocytosis, a pathway that is used in the absorption of orally administered chondroitin sulfate.[37] Based on the Caco-2 transfer, we infer that FCS oligosaccharides are much easier to be absorbed in the intestine in vivo than FCS polysaccharides. The high absorption efficiency of FCS oligomers into the plasma can make up for their weaker anticoagulant activity than that of native FCS, resulting in a promising anticoagulant effect.

We have provided detailed information on the FCS oligomers and native FCS, including their structure as well as the in vitro anticoagulant properties, in previous publications.[23,38,39] Both FCS polysaccharides and oligosaccharides are resistant to the vertebrate enzymes, such as hyaluronidase and chondroitinases, due to their unique sulfated fucose branches.[14,40] Thus, neither of these anticoagulants would be enzymatically digested during passage through the intestinal tract. The native FCS and FCS

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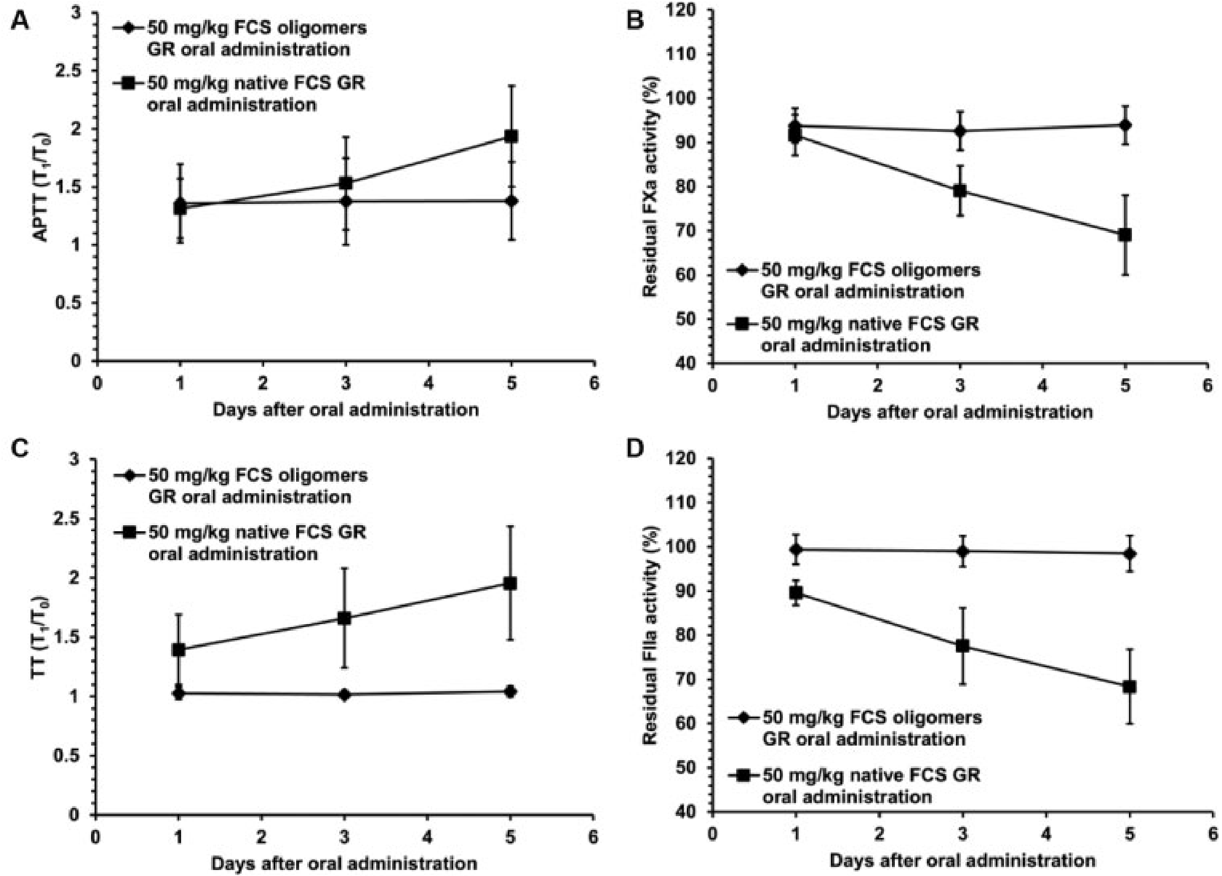

Fig. 6 The APTT (**A**) and TT (**C**) as well as the residual activities of FXa (**B**) and FIIa (**D**) during continuous oral administration of FCS oligomer-GR microcapsules or native FCS-GR microcapsules. Plasma samples were collected 5 hours after the first administration, on the first, third, and fifth days. The APTT and TT as well as the residual activities of FXa and FIIa were measured and expressed as previously (mean ± SD, $n = 5$). APTT, activated partial thromboplastin time; FIIa, factor IIa; FXa, factor Xa; FCS, fucosylated chondroitin sulfate; GR, gastro-resistant; SD, standard deviation; TT, thrombin time.

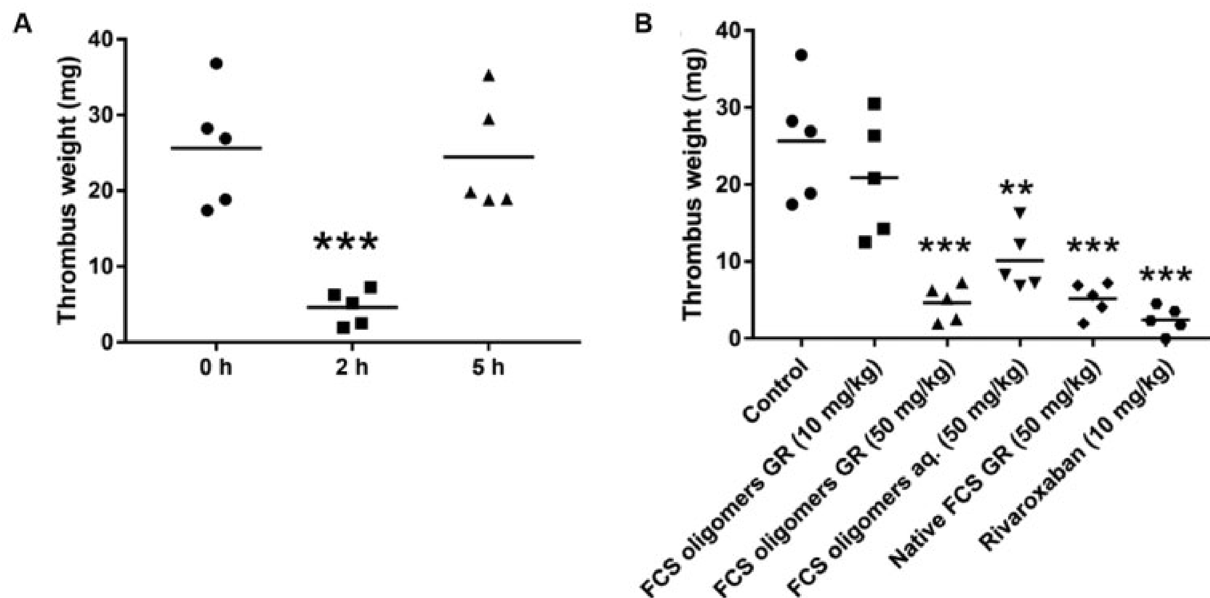

Fig. 7 (**A**) Antithrombotic effects at 2 and 5 hours after oral administration of 50 mg/kg FCS oligomer-GR microcapsules. (**B**) Antithrombotic effects at 2 hours after oral administration of 10 or 50 mg/kg FCS oligomer-GR microcapsules, 50 mg/kg FCS oligomers in aqueous solution (aq.), 50 mg/kg native FCS-GR microcapsules, and 10 mg/kg rivaroxaban. Antithrombotic activity was investigated in male Sprague Dawley rats with the rabbit brain thromboplastin-induced venous thrombosis model. The results were expressed as thrombus weight (mean ± SD, $n = 5$, $^{**}p < 0.01$, $^{***}p < 0.01$ vs. control). FCS, fucosylated chondroitin sulfate; GR, gastro-resistant; SD, standard deviation.

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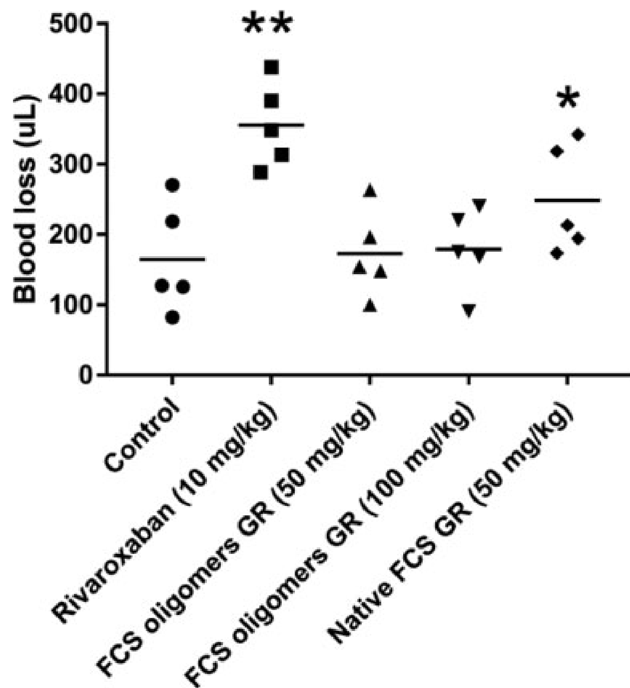

Fig. 8 Bleeding risk of different anticoagulants.10 mg/kg rivaroxaban, 50 or 100 mg/kg FCS oligomer-GR microcapsules, and 50 mg/kg native FCS-GR microcapsules were orally administrated into rats. Two hours later, the rat tail was cut 5 mm from the tip and immersed in 40 mL of distilled water at 37°C. Blood loss was determined by measuring the hemoglobin present in the water using a spectrophotometric method. The results were expressed as microliters of blood loss (mean ± SD, $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control). FCS, fucosylated chondroitin sulfate; GR, gastro-resistant; SD, standard deviation.

oligomers in plasma exhibit the same anticoagulant properties as they do in vitro. FCS oligomers in plasma could only prolong the APTT and reduce FXa activity. In contrast, native FCS could prolong APTT and TT as well as reduce the FXa and FIIa activities. This suggests that oral administration of native FCS-GR microcapsules may cause some side effects due to their action on multiple targets in the coagulation pathway.

In a previous study, a kind of FCS oligosaccharides (average $M_w \approx 12$ kDa) was almost completely cleared from the plasma in approximately 6 hours after *intravenous* administration.[41] In this study, the FCS oligomers in plasma delivered by oral administration of the GR microcapsules or aqueous solution exhibited a similar cleared tendency as reflected by the APTT. Meanwhile, continuous oral administration of FCS oligomer-GR microcapsules every 12 hours did not cause accumulation of oligosaccharides in the plasma. However, the continuous oral administration of native FCS-GR microcapsules every 12 hours would cause accumulation of polysaccharides in plasma. It indicated that FCS polysaccharides were cleared much more slower than oligosaccharides under continuous oral administration. Since the FCS polysaccharides or oligosaccharides in plasma were most probably cleared through the kidney,[41] it is critical to ascertain the influence of FCS polysaccharides or oligosaccharides on the kidney during the clearance in future studies.

In conclusion, FCS oligomers delivered by oral administration from GR microcapsules could prevent structural damage caused by stomach acid and exhibit a slow release in the intestinal tract, resulting in prolonged anticoagulant and antithrombotic effects in the plasma. Moreover, FCS oligomers exhibited greater absorption efficiency than native FCS polysaccharides in a Caco-2 system. Native FCS polysaccharides exhibited much stronger anticoagulant and antithrombotic properties than FCS oligomers. However, FCS polysaccharides in plasma could cause bleeding tendency, even at a low content, due to their action on multiple targets in the coagulation pathway. FCS oligomers, a mixture of FCS 6 to 18 saccharide units, exhibited selective inhibition of intrinsic FXase in vitro and in vivo. The specific anticoagulant activity of the FCS oligomers in plasma provided potent in vivo antithrombotic activity while avoiding side effects such as bleeding. Our results suggest that FCS oligomer-GR microcapsules represent a promising oral anticoagulant for clinical use.

What is known about this topic?

- FCS, extracted from sea cucumber, is a potent anticoagulant polysaccharide attributed to the presence of unique sulfated fucose branches.
- This glycosaminoglycan is resistant to the enzymes present in vertebrates so that it could be administrated orally as GR tables to exhibit remarkable anticoagulant and antithrombotic activities in vivo.
- However, bleeding could occur after its oral administration due to the multiple plasma targets of FCS polysaccharide in the coagulation pathway.
- FCS oligomers including 6 to 18-mer have shown potent anticoagulant and antithrombotic activities by selectively inhibiting intrinsic FXase.

What does this paper add?

- We prepared FCS oligomer-GR microcapsules as oral anticoagulation and the FCS oligomers showed remarkable absorption without structure modification in a Caco-2 model.
- Oral administration of FCS oligomers in GR microcapsules exhibited potent antithrombotic activity by inhibiting intrinsic coagulation pathway and no bleeding risk in vivo.
- Continued oral administration of FCS oligomer-GR microcapsules did not result in the accumulation of oligosaccharides in the plasma.
- Our work suggested that a FCS oligomer-GR microcapsule formulation represents an effective and safe oral anticoagulant for potential clinical applications.

Authors' Contributions

L.Y., M.Z., D.W., and W.T. performed the experiments; L.Y., X.Y., and F.Z. analyzed the results; and L.Y., R.J.L., and S.C. wrote the manuscript.

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Conflict of Interest
None declared.

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