

Optimized alkylated cyclodextrin polysulphates with reduced risks on thromboembolic accidents improve osteoarthritic chondrocyte metabolism

Sara Groeneboer¹, Stijn Lambrecht¹, Aad Dhollander¹, Peggy Jacques¹, Bert Vander Cruyssen¹, Rik J. Lories², Katrien Devreese³, Koen Chiers⁴, Dirk Elewaut¹ and Gust Verbruggen¹

Abstract

Objectives. To compare the ability of different cyclodextrin polysulphate (CDPS) derivatives to affect human articular cartilage cell metabolism *in vitro*.

Methods. OA chondrocytes were cultured in alginate and exposed to 5 µg/ml of 2,3,6-tri-*O*-methyl-β-cyclodextrin (ME-CD), 2,3-di-*O*-methyl-6-sulphate-β-cyclodextrin (ME-CD-6-S), 2,6-di-*O*-methyl-3-sulphate-β-cyclodextrin (ME-CD-3-S), (2-carboxyethyl)-β-CDPS (CE-CDPS), (2-hydroxypropyl)-β-CDPS (HP-CDPS), 6-monoamino-6-monodeoxy-β-CDPS (MA-CDPS) or β-CDPS for 5 days. Effects on IL-1-driven chondrocyte extracellular matrix (ECM) metabolism were assayed by analysis of the accumulation of aggrecan in the interterritorial matrix, IL-6 secretion and qPCR. MA-CDPS, HP-CDPS, CE-CDPS and CDPS were analysed for their *in vitro* effect on coagulation and their ability to activate platelets in an *in vitro* assay to detect possible cross-reactivity with heparin-induced thrombocytopenia (HIT) antibodies.

Results. The monosulphated cyclodextrins ME-CD-6-S and -3-S failed to affect aggrecan synthesis and IL-6 secretion by the OA chondrocytes. Polysulphated cyclodextrins MA-CDPS, HP-CDPS, CE-CDPS and CDPS at 5 µg/ml concentrations, on the other hand, significantly induced aggrecan production and repressed IL-6 release by the chondrocytes in culture. aPTT and PT for all derivatives were lengthened for polysaccharide concentrations >50 µg/ml. Five micrograms per millilitre of β-CDPS concentrations that significantly modulated ECM ground substance production *in vitro* did not affect aPTT or PT. Furthermore, CE-CDPS, in contrast to MA-CDPS, HP-CDPS and CDPS, did not significantly activate platelets, suggesting a minimal potential to induce HIT thromboembolic accidents *in vivo*.

Conclusions. CE-CDPS is a new, structurally adjusted, sulphated β-cyclodextrin derivative with preserved chondroprotective capacity and a promising safety profile.

Key words: Chondroprotection, Osteoarthritis, Cyclodextrin polysulphates.

Introduction

OA is characterized by the degeneration and eventual loss of cartilage with concurrent changes in the subchondral bone. While it is generally accepted that mechanical stress may result in the damage that will set off the OA process, its progression involves the activities of multiple auto/paracrine cytokine activities. IL-1 is believed to be a major regulator of matrix degradation by increasing metalloproteinase activity and suppressing the synthesis of matrix components. In addition to significantly higher intracellular levels of IL-1β, chondrocytes from fibrillated cartilage show an up-regulation of the signalling IL-1

¹Department of Rheumatology, Laboratory of Connective Tissue Biology, Ghent University Hospital, Ghent, ²Department of Musculoskeletal Sciences, Division of Rheumatology, Laboratory for Skeletal Development and Joint Disorders, KU Leuven, ³Department of Clinical Chemistry, Microbiology and Immunology, Coagulation Laboratory, Ghent University Hospital and ⁴Department of Pathology, Bacteriology and Avian Diseases, Ghent University, Ghent, Belgium.

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Correspondence to: Gust Verbruggen, Department of Rheumatology, Laboratory of Connective Tissue Biology, Ghent University Hospital, De Pintelaan, 185 BE-9000, Ghent, Belgium. E-mail: gust.verbruggen@ugent.be

receptor I (IL-1RI) levels compared with normal cartilage [1]. Despite the attempt of chondrocytes to restore the homeostasis of their matrix by enhancing insulin-like growth factor 1 (IGF-1) and TGF- β growth factor activity [2, 3], the catabolic activity of the OA chondrocyte results in a continuous net loss of matrix components ensuing disease progression.

The search for agents that restore the structural deficiencies underlying OA started in the mid-1970s with the findings that sulphated polysaccharides, e.g. heparin and chondroitin sulphates, with varying degrees of sulphating, enhanced the synthesis of extracellular matrix (ECM) substances by connective tissue cells in culture [4, 5]. In particular, the polysulphated polysaccharides provided significant effects on the structure and function of articular cartilage cells in culture [6, 7]. These effects were also observed *in vivo* [8–10]. Consequently, both xylosan polysulphate and chondroitin polysulphate found their place in the treatment of osteoarticular pathology in veterinary and in human medicine, respectively.

Apart from the chondroprotective capacities, however, these polysulphated polysaccharides were revealed to possess important biological activities similar to those of heparin. Such biological activities were clearly shown to be related to the molecular structure of the polysaccharide and varied following distinct modifications. *In vitro* and *in vivo* studies on the effects of these polysaccharides on coagulation showed variable effects on thrombin clotting times. It was, however, the possibility of such polysulphated polysaccharides to induce heparin-induced thrombocytopenic thrombosis (HITT) through cross-reaction with heparin/platelet factor-4 antibodies that had raised serious concern [11]. These antibodies arise occasionally when activated thrombocytes release platelet factor-4 (PF4) during heparin treatment. Heparin then forms a complex with PF4 that acts as an antigen which triggers the production of auto-antibodies. These antibodies bind to the complex via their F(ab) region and to the low affinity immunoglobulin gamma Fc region receptor II-b (Fc γ RII) [immunoglobulin G (IgG) CD32] of other platelets via their Fc portion, thereby initiating platelet activation, aggregation and thromboembolic accidents [12]. It has been shown that some low-molecular weight heparins as well as other sulphated polysaccharides, e.g. chondroitin polysulphates [13], can also bind to heparin-induced thrombocytopenia (HIT) antibodies in the presence of PF4 and that their reactivity is dependent on their molecular weight and the sulphating grade [12, 14, 15].

When, in the 1980s, controversial reports on the occurrence of HITT following treatment with chondroitin polysulphate came out [13], national and international agencies for the evaluation of medicinal products insisted that the previous manufacturer of this polysulphated chondroitin (Arteparon; Luitpold Werk, München, Germany) provided the conventional drug master files confirming the claims on efficacy and safety of that drug in human clinics. As the patent life of chondroitin polysulphate had gone, the manufacturer at that time did not respond to this demand and the European formulation of chondroitin polysulphate

was taken off the market in 1994. Neutraceutical industries then introduced the naturally occurring chondroitin sulphate to replace chondroitin polysulphate for the use in degenerative joint disease in humans. In the sole head-to-head confrontation thus far, the naturally occurring chondroitin sulphate was inferior to chondroitin polysulphate when the chondroprotective effects of both drugs were assessed in a population with hand OA [16].

Recently, a novel polysulphated polysaccharide, cyclodextrin polysulphate (CDPS) was reported to induce a down-regulation of intracellular IL-1 α and - β and to cause a concomitant increase in the synthesis of aggrecan, collagen Type II and fibronectin in the cell-associated matrix (CAM) of human chondrocytes cultured in alginate beads [17]. This CDPS also depressed the IL-6 release of OA chondrocytes by 60% compared with untreated OA cartilage cells and equalled the levels secreted by normal cells [17].

CDPS, subcutaneously administered in a rabbit model of experimental OA, reduced the cartilage lesions and osteophyte formation in the affected joints [18]. These data suggest that CDPS positively affects the tissue pathology underlying OA and this agent can therefore be classified as a structure or disease-modifying OA drug.

To alleviate possible heparin-related side effects, we have developed six sulphated β -cyclodextrin derivatives by introducing hydrophobic substituents on the 2, 3 and/or 6 position, assuming they would preserve their chondroprotective effect. Next, the effect on coagulation and the potency to induce thrombocytopenia through cross-reaction with heparin/PF4 antibodies were assayed. Once optimized, these derivatives were tested for their capacity to restore cartilage damage *in vitro*.

Materials and methods

β -Cyclodextrin derivatives

The 2,3,6-tri-O-methyl- β -cyclodextrin (ME-CD) was purchased from Cyclolab Ltd (Budapest, Hungary) and served as a non-sulphated control molecule. β -Cyclodextrins carrying one sulphate on each glucopyranose unit included 2,3-di-O-methyl-6--sulphate- β -cyclodextrin (ME-CD-6-S: Regis Technologies Inc.; Morton Grove, IL, USA) and 2,6-di-O-methyl-3-sulphate- β -cyclodextrin (ME-CD-3-S: Cyclolab Ltd). Commercially available (2-carboxyethyl)- β -cyclodextrin (CE-CD containing, on average, three carboxyethyl groups: Sigma Chemical Company, St Louis, MO, USA), (2-hydroxypropyl)- β -cyclodextrin (HP-CD containing seven hydroxypropyl groups: Sigma Chemical Company) and 6-monodeoxy-6-monoamino- β -cyclodextrin (MA-CD having one -OH function replaced by -NH₂; Cyclolab Ltd) were used to synthesize the polysulphated β -cyclodextrins or sulphaalkyl- β -cyclodextrins.

Sulphating was achieved according to the procedure described by Astrup *et al.* [19]. The degree of substitution (DS) of the β -cyclodextrin derivatives was characterized by elemental analysis of C, N, H and S. The ratio of sulphur to carbon percentages was used to

determine the DS of the β -cyclodextrin glucopyranose unit: $DS = [\%S \times m_a (C) / \%C \times m_a (S)] \times \text{total no. of C}$, where m_a is the atomic mass (Table 1).

Isolation of articular chondrocytes

Human articular chondrocytes were isolated as described elsewhere [20]. Briefly, human articular cartilage was obtained at total knee replacement surgery from femoral condyles and the tibial plateau of 15 different donors. None of them had received CSs or cytostatic drugs. Both visually intact and fibrillated OA cartilage samples were harvested separately and only OA cartilage was used for cell culture as OA progression involves the activities of auto/paracrine IL-1 activity [1] and previous experiments revealed definite effects of polysulphated polysaccharides on IL-1-primed chondrocytes after their isolation from normal cartilage [7]. The cartilage samples were diced into small fragments and the chondrocytes were isolated by sequential enzymatic digestion with hyaluronidase, pronase and collagenase. Isolated cells were then centrifuged for 10 min at 524 g, washed three times in DMEM with 10% (v/v) fetal calf serum (FCS) and counted with Trypan blue to exclude dead cells. More than 90% of the cells were viable after isolation. The study was approved by the Ethical Committee of Ghent University Hospital. Informed consent was obtained from the patients.

Cultures in alginate gel

Chondrocytes were cultured in alginate beads to maintain their differentiated phenotype. The cultures were prepared as described elsewhere, with some modifications [20, 21]. Chondrocytes suspended in one volume of double-concentrated Hank's balanced salts solution (HBSS) without calcium and magnesium (Gibco, Life Technologies, Paisley, UK) were carefully mixed with an equal volume of 2% (w/v) autoclaved alginate (low-viscosity alginate from *Macrocystis pyrifera*; Sigma) in HBSS. The final cell concentration was 5×10^6 chondrocytes/ml in 1% alginate. The chondrocyte/alginate suspension was then slowly dripped through a 23-gauge needle into a 102-mM calcium chloride solution. The beads were allowed to polymerize for 10 min at room temperature.

After removal of the calcium chloride, the beads were washed three times with 0.15 M sodium chloride. Alginate beads were cultured in 12-well plates with 1×10^6 cells per culture (each well containing 20 alginate beads; $\pm 50\,000$ chondrocytes per bead) in 3 ml DMEM supplemented with 10% FCS and 50 mg ascorbate/ml at 37°C under 5% CO₂. Nutrient medium was replaced twice weekly. It has been shown that ECM metabolism by chondrocytes reaches steady state after 1 week in this alginate culture system [22].

IL-6 release and ECM synthesis by chondrocytes after treatment with the differently sulphated β -cyclodextrins *in vitro*

Cartilage cells obtained from OA cartilage were used to evaluate the effect of the different β -cyclodextrin polysulphates on the synthesis and accumulation of ECM aggrecan. Fifteen donor samples allowed seven polysaccharides to be compared 6–11 times with the control situation. At Day 5 of culture, the OA chondrocytes of each donor were exposed to 5 μ g/ml of, respectively, ME-CD, ME-CD-3-S, ME-CD-6-S, 6-monoamino-6-monodeoxy- β -CDPS (MA-CDPS), (2-hydroxypropyl)- β -CDPS (HP-CDPS), (2-carboxyethyl)- β -CDPS (CE-CDPS) and CDPS. After 5 additional culture days, media were collected and stored at -20°C . The cells were separated from their alginate coat by dissolving the alginate with 3 ml of 55-mM tri-sodium citrate dehydrate pH 6.8, 0.15 M NaCl at 25°C for 10 min. The resulting suspension was centrifuged at 524 g for 10 min to separate the supernatant containing the constituents of interterritorial matrix (IM) from the cells with their CAM. The aggrecan content (Biosource, Merelbeke, Belgium) and IL-6 (R&D Systems, Abingdon, UK) release in the IM was assayed by ELISA according to the manufacturer's instructions. All experiments were performed in triplicate. Chondrocytes from four additional patients [mean (s.d.) 63.5 (9.3) years] were isolated and cultured as described above. After the culture period, Trizol (Invitrogen, Life Technologies, Paisley, UK) was added to the isolated cells. RNA was extracted and cDNA prepared as previously described [23]. Real-time PCR was performed using the ABI 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA,

TABLE 1 Chemical analysis of the different β -cyclodextrins

β -Cyclodextrin	Elemental analysis				S-substitution	DS/glucopyranose
	C, %	H, %	N, %	S, %		
ME-CD						0.00 ^a
Heptakis ME-CD-3-S						1.00 ^a
Heptakis ME-CD-6-S	28.66	4.50	<0.05	9.77	7.12	1.02
Sulphated MA-CDPS	16.11	2.78	0.47	16.34	15.96	2.28
Sulphated CE-CDPS	16.80	2.63	<0.05	15.33	17.34	2.47
Sulphated HP-CDPS	18.91	3.28	<0.05	16.40	20.48	2.92
CDPS	17.28	2.86	<0.05	16.94	15.41	2.21

^aManufacturer's data. S-substitution: number of sulphate groups per β -cyclodextrin; DS/glucopyranose: sulphate groups per β -cyclodextrin glucopyranose unit.

USA). Each reaction utilized 5 μ l of cDNA and a mixture of 20 μ l of iTaq Sybr Green Supermix with Rox (Bio-Rad, Hercules, CA, USA), primers (Invitrogen) and water and was performed in triplicate. The following primer sequences were used: aggrecan Fw: TGGTCAAAGGCATC-GTGTTT, aggrecan Rev: GTGGCAATGATGGCACTGTT; Col2A1 Fw: GGCAATAGCAGGTTCCACGTACA, COL2A1 Rev: CGATAACAGTCTTGCCCCACTT; BMP-2 Fw: CCA-ACACTGTGCGCAGCTT, BMP-2 Rev: AAGAATCTCCGG-GTTGTTTTCC; TIMP1 Fw: TGACATCCGGTTCGTCTACA, TIMP1 Rev: TGCAGTTTTCCAGCAATGAG; Col1A1 Fw: TGCCATCAAAGTCTTCTGCAA, COL1A1 Rev: CGCCAT-ACTCGAAGTGAATC; SOX-9 Fw: GCTCAGCAAGACG-CTGGGCA, SOX-9 Rev: ATGTGCGTCTGCTCCGTGGC; MMP2 Fw: CTACGATGGAGGCGCTAATG, MMP2 Rev: GGCAGCCATAGAAGGTGTTT; ADAMTS5: TaqMan Gene expression Assay (ABI). All primer sequences were validated by melting curve analysis and efficiency analysis. qPCR and data analysis were performed as described earlier [23, 24].

β -Cyclodextrins and blood coagulation activities

MA-CDPS, HP-CDPS, CE-CDPS and CDPS were prepared as buffered solutions at various concentrations. The polysaccharides were incubated with normal pooled plasma, prepared out of 40 healthy volunteers and analysed for their effects on aPTT, PT and fibrinogen levels on an STA Compact[®] (Diagnostics Stago, Asnières, France) coagulation analyser according to the manufacturer's instructions.

β -Cyclodextrins and HIT

The activation of healthy donor thrombocytes by HIT patient anti-heparin/PF4 antibodies in the presence of heparin and the differently sulphated forms of β -cyclodextrin was tested. Plasma of patients that experienced HIT thromboembolic accidents following the administration of heparin and which showed anti-heparin/PF4 antibodies (HIT Abs) on immunological and flow cytometric tests [25–27] were selected for these assays. The expression of CD62p by the activated platelets was assayed by means of flow cytometry with a FC500 Beckman Coulter (Beckman Coulter, Anlis, Belgium).

Platelet-rich plasma (PRP) solution was prepared by slow centrifugation (10 min at 180 g) of fresh citrate anti-coagulated blood (1 : 9 v/v, 0.129 mol/l or 3.8% tri-sodium citrate buffer), obtained from normal donors. HIT Ab-positive plasma samples from patients and HIT Ab-negative control plasma from healthy donors were prepared by double centrifugation (15 min at 1157 g) of fresh citrate anti-coagulated blood. Seventy microlitres of PRP was incubated with 20 μ l of HIT Ab containing plasma in the presence of different concentrations of heparin (0 and 0.3 IU/ml) or the different β -cyclodextrins (0 and 5 μ g/ml). Experiments with each HIT Ab-positive plasma sample were repeated independently using thrombocytes of two donors (A and B), to avoid the possibility of non-reactive platelets.

Following the initial 40-min incubation step, 5 μ l of the platelet solution were transferred to a fresh tube with 85 μ l PBS containing 1% BSA and 0.1% sodium azide, 5 μ l (0.25 μ g) monoclonal anti-CD41 Abs [phycoerythrin (PE)-Texas Red energy coupled dye (ECD)-conjugated, Beckman Coulter] and 5 μ l (0.125 μ g) of anti-CD62p Ab (PE-conjugated, Beckman Coulter). CD41 (glycoprotein IIb) is a selective marker of platelets and platelet precursors. CD62p is a constituent of α -granules and is released to the platelet surface on activation [12, 14].

After 20 min of incubation at room temperature, the total volume of the platelet suspension was adjusted to 600 μ l buffered solution and evaluated without delay by flow cytometry. Twenty thousand events per sample were analysed and the platelet populations were gated by side scatter characteristics and the platelet marker CD41. Activated platelets (CD62p⁺) were distinguished from resting platelets by the CD62p-PE expression (Fig. 1). The fraction of activated platelets among the total platelet population was determined and relative CD62p ratios to the internal controls were calculated. [ratio: %CD62p⁺ (0.3 IU heparin or 5 μ g/ml β -cyclodextrin derivate)/%CD62p⁺ (0 IU heparin or 0 μ g/ml β -cyclodextrin derivate)]. Test results for the heparin-treated platelets were evaluated by a scoring system adapted from Jy *et al.* [14]. Control tests were scored positive when the calculated ratios of at least two donors were >2, which is based on previously obtained results with normal individuals.

To evaluate the potential of different β -cyclodextrins to induce platelet activation, both the percentages and ratios of CD62p⁺ platelets after incubation with the respective β -cyclodextrin were statistically compared with the percentage and the ratio of CD62p⁺ platelets after heparin incubation. The number of normal individuals who were tested with the β -cyclodextrin derivatives was too small to set a correct cut-off for these ratios.

Statistical analysis

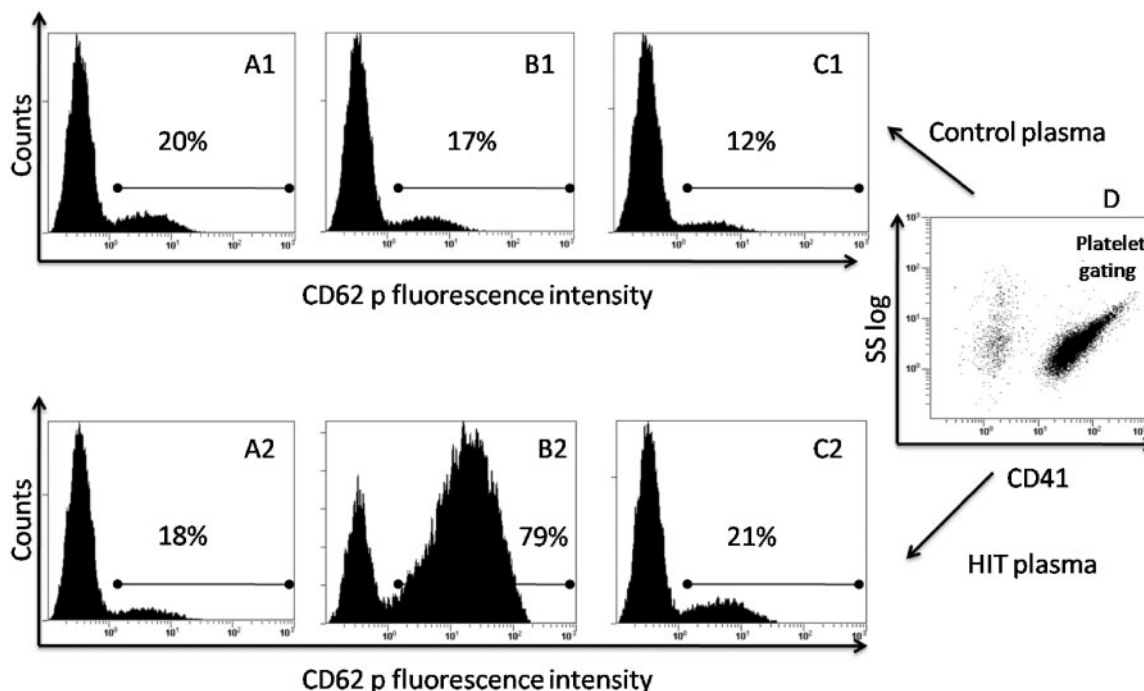
To study the impact of the changes in the chondrocyte cultures after CDPS treatment, mean IL-6 and aggrecan concentrations were calculated from triplicate chondrocyte cultures of each donor. Student's *t*-tests were used to test significance of differences between untreated controls and cultures after exposure to different CDPSs. Cross-reactivity with heparin of the polysaccharides on *in vitro*-induced platelet activation was analysed by Wilcoxon signed-rank test.

Results

Chemical analysis of the different β -cyclodextrins

From the data provided by the elemental analysis of the different β -cyclodextrin preparations (Table 1), ME-CD-6-S, as ME-CD-3-S, appeared to contain one sulphate group per glucopyranose unit. Sulphating occurred at positions 6 and 3 of each glucopyranose unit in ME-CD-6-S and ME-CD-3-S, respectively. HP-CDPS showed 2.92 sulphate groups per glucopyranose. Therefore, two

Fig. 1 *In vitro* activation of platelets by HIT ab-positive plasma with heparin. Percentages of cell staining with CD62p after addition of 5 μ l heparin (**A1** and **A2**: 100 IU/ml; **B1** and **B2**: 0.3 IU/ml; **C1** and **C2**: 0 IU/ml) in the absence (**A1**, **B1** and **C1**) or presence (**A2**, **B2** and **C2**) of HIT ab-positive plasma. Platelets gated on side scatter (SS log) and CD41 activity (**D**). The interface channel for positivity was set at the point where <2% of the control fluorescence was positive.



glucopyranose OH-functions, as well as the OH-function of the hydroxypropyl group, have been substituted by sulphate. Equally, CE-CDPS with glucopyranoses containing 2.47 sulphate groups had all remaining glucopyranose OH-functions substituted by sulphate. Although the sulphating procedure should have resulted in 3-sulphated glucopyranoses, degrees of sulphating of 2.28 and 2.21 of each glucopyranose were calculated for MA-CDPS and CDPS, respectively. To conclude, the *in vitro* tests were done with one unsulphated cyclodextrin: ME-CD, two monosulphated cyclodextrins sulphated on different C atoms: ME-CD-3-S and -6-S and 4 CDPSs with degrees of sulphating ranging from 2 to 3: CDPS, MA-, CE- and HP-CDPS. Chemical structures of the different CDPS are given in Fig. 2.

Effects on ECM production and inhibition of IL-6 release by differently sulphated β -cyclodextrins

The unsulphated ME-CD failed to promote aggrecan synthesis or to affect IL-6 release and was used as a control in the experiments on isolated chondrocytes. Furthermore, the monosulphated cyclodextrins ME-CD-3-S and -6-S also failed to affect chondrocyte aggrecan synthesis and IL-6 secretion. The bisulphated (CDPS, MA-CDPS) and trisulphated β -cyclodextrins (HP-CDPS, CE-CDPS), however, significantly enhanced chondrocyte aggrecan synthesis. When compared with the controls, average percentage increases of 51.6 ($P=0.009$), 41.0% ($P=0.006$), 30.4% ($P=0.020$) and 67.3%

($P=0.007$) for CDPS, MA-CDPS, HP-CDPS and CE-CDPS, respectively, were noted (Fig. 3A). These highly sulphated β -cyclodextrins significantly repressed chondrocyte IL-6 secretion by $\sim 25\%$ with $P < 0.001$ for the four polysaccharides tested (Fig. 3B). Based on the results of the blood coagulation and HIT-analysis, CE-CDPS was selected for further analysis on chondrocyte metabolism. qPCR analyses were performed of additional ECM genes (*COL2A1*, *aggrecan*, *COL1A1*), catabolic enzymes and enzyme inhibitors (ADAMTS5, MMP2, TIMP-1), transcription factors (SOX-9) and growth factors (Bone Morphogenetic Protein 2) to provide further insights into the effect of CE-CDPS on chondrocyte metabolism. In parallel, with the ELISA analysis mentioned above, aggrecan gene expression was elevated in three of four patients analysed. No effects were observed on collagen expression (*COL2A1* and *COL1A1*). The MMP-2 enzyme showed a lower expression upon stimulation with CE-CDPS in three patients (in one of the patients no expression of MMP2 could be detected). Upon CE-CDPS stimulation, a significant elevated expression was observed for the transcription factor SOX-9 (on average 3.2-fold induction), confirming the positive effect of CE-CDPS on the chondrocyte's anabolic activity (Fig. 3C-E).

β -Cyclodextrin effects on blood coagulation activities

With increasing concentrations, the aPTT values stagnated up to a concentration of 5 μ g/ml for all the CDPS derivatives studied. Above 50 μ g/ml no clot formed for the

Fig. 2 Chemical structures of β -cyclodextrin derivatives.

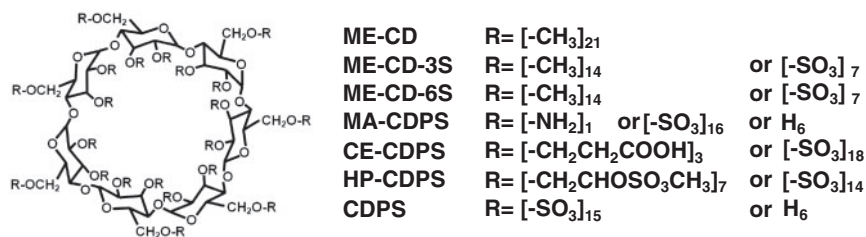
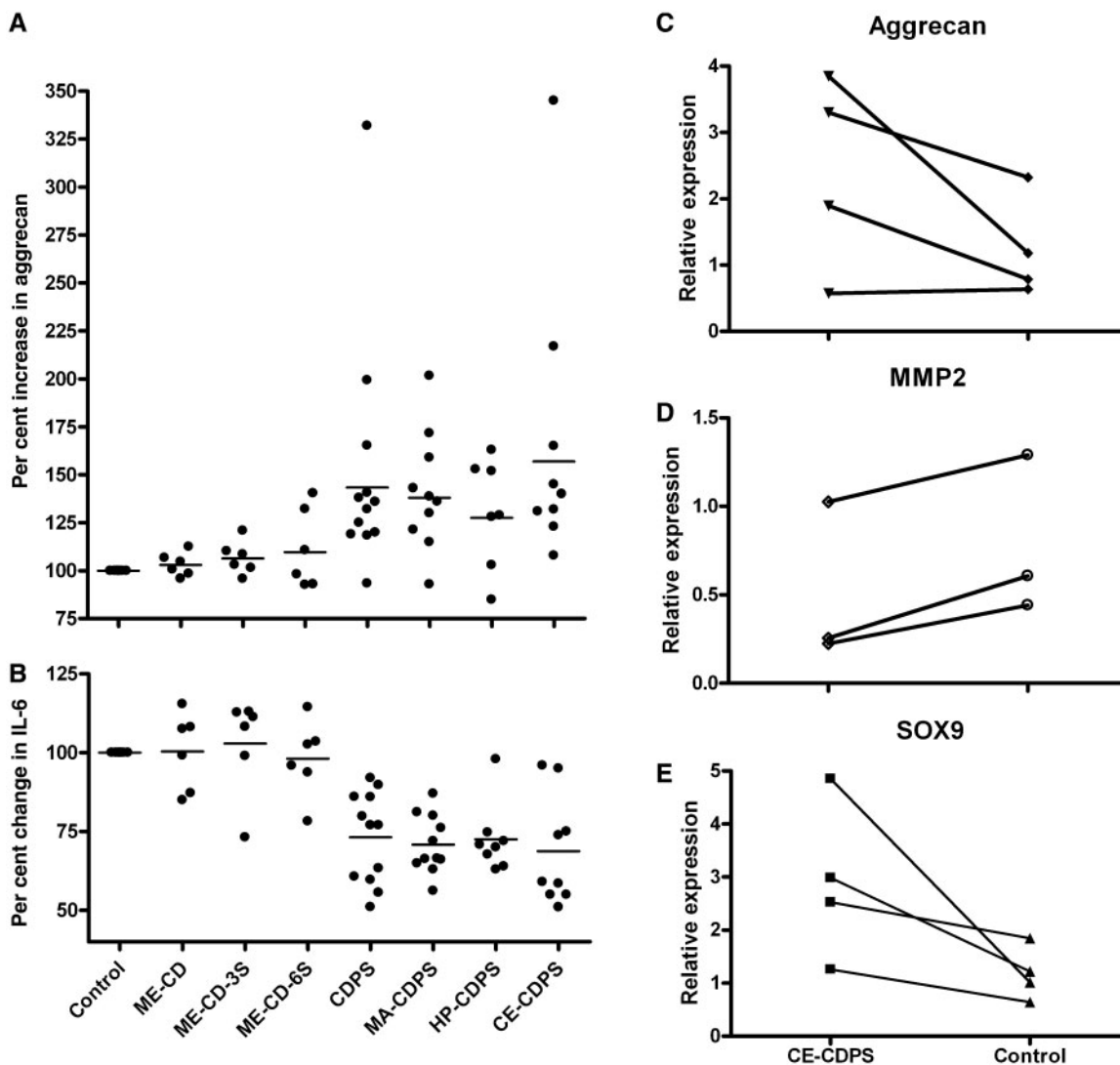


Fig. 3 (A) Percentage changes in aggrecan production by OA chondrocytes treated with β -CDPS derivatives. Dots represent mean values of triplicate cultures of one patient. Lines represent the overall mean. (B) Percentage changes in IL-6 release by OA chondrocytes treated with β -CDPS derivatives. Dots represent mean values of triplicate cultures of one patient. Lines represent the overall mean. (C-E) Relative expression of aggrecan, MMP2 and SOX-9, respectively, as determined by qPCR. Four additional patient samples were stimulated with CE-CDPS and compared with controls. Dots represent the mean expression of duplicate cultures. No expression of MMP2 was observed in one of the samples.

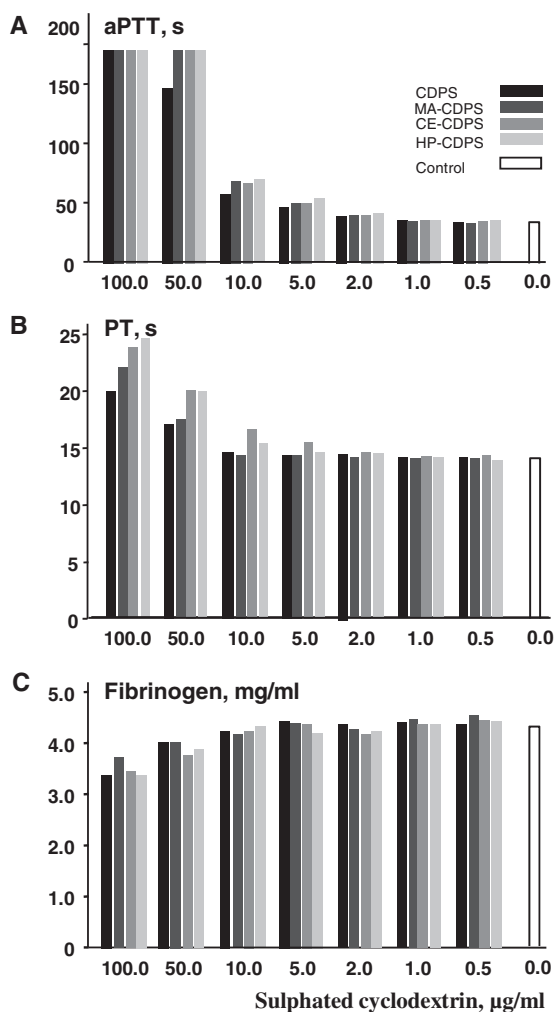


derivatives studied in the observation time of the experiment (Fig. 4A). The same tendency was observed for PT values, except for the far more limited increase in PT for concentrations $>50\ \mu\text{g/ml}$ (Fig. 4B). Minor decreases were observed in fibrinogen concentrations (mg/ml) after incubation with 50 or $100\ \mu\text{g/ml}$ of MA-CDPS, HP-CDPS, CE-CDPS or CDPS, respectively (Fig. 4C). No influence on the blood coagulation cascade could thus be detected at concentrations that highlighted a chondroprotective effect *in vitro*.

Induction of platelet activation

All HIT Ab-positive plasma samples showed platelet heparin activation ratios $\%CD62p^+$ (0.3 IU heparin)/ $\%CD62p^+$ (0 IU heparin) >2 for the two donors, indicating an activation of platelets by heparin/PF4 antibodies.

Fig. 4 Effects of the β -CDPS derivatives on variables of plasma coagulation activity in a pool of 40 serum samples. The assays were done once as the results of these types of tests are particularly consistent. (A) aPTT, in s; (B) PT, in s; and (C) fibrinogen, in mg/ml; abscissa: sulphated cyclodextrins, in $\mu\text{g/ml}$.



Platelets were not activated by heparin or any of the β -CDPS derivatives in the absence of patient's HIT Ab (data not shown).

Immunological cross-reactivity between heparin and some of the polysulphated β -cyclodextrins became evident as HP-CDPS, MA-CDPS and CDPS induced platelet activation in the presence of the plasma samples of the individuals that had developed HIT Ab upon previous exposure to heparin (Fig. 1). Paired analysis of both ratios and percentages of $CD62p^+$ cells confirmed a significant difference ($P=0.017$ for percentages and $P=0.012$ for ratios) between heparin and CE-CDPS-incubated platelets, indicating that CE-CDPS exhibited no cross-reactivity with the heparin/PF4 antibodies (Table 2). Moreover, the P -values calculated from the paired analysis of ratios and percentages of activated platelets between CE-CDPS and each of the other analysed cyclodextrins was <0.05 .

Discussion

Among the polysulphated polysaccharides shown to improve connective tissue cell ECM metabolism *in vitro* and *in vivo* [4–10], chondroitin polysulphate was mainly found to be applicable in the treatment of osteoarticular pathology. The safety of this drug in human clinics, however, became controversial. The purpose of this study was, therefore, to search for more advanced polysaccharide polysulphates with a preserved chondroprotective capacity, a reduced effect on coagulation and, imperatively, a reduced risk for HIT.

The structure-modifying capacities of CDPS have been evidenced previously. *In vitro*, all three α -, β - and γ -CDPS equally and significantly improved the synthesis and accumulation by chondrocytes of CAM macromolecules e.g. aggrecan, Type II collagen and fibronectin [17]. It was shown that this enhanced repair function resulted from a down-modulation of the autocrine catabolic IL-1 pathway: β -CDPS inhibited the synthesis of IL-6 in both normal and OA chondrocytes. Although the experiments were not performed in the presence of IL-1-blocking antibodies, it is supposed that β -cyclodextrin affected the IL-1 loop and did not directly impede the production of IL-6 [17]. While in osteoarthritic chondrocytes both autocrine anabolic (IGF-1) and catabolic (IL-1) pathways are up-regulated, the selective inhibition of IL-1 resulted in a dramatic improvement of the repair function. *In vivo*, this repair-promoting effect was obvious when rabbits, in which the anterior cruciate ligament of the knees were sectioned, failed to develop cartilage degradation when treated with s.c. injections of 1 mg/kg γ -CDPS once weekly [18]. In these *in vivo* experiments, however, a 15-times higher dose (5 mg/kg s.c. three times weekly) caused haemorrhages in some of the treated animals and a 90-times higher dose (30 mg/kg s.c. three times weekly) even caused fatal haemorrhages in most animals [18]. A series of polysulphated cyclodextrin derivatives were thus synthesized to come across potentially less harmful treatments. β -Cyclodextrins with different alkyl substitutions on distinct $-\text{OH}$ groups allowed for the

TABLE 2 Platelet activation by heparin and polysulphated β -cyclodextrin derivatives

Treatment	Patient 1		Patient 2		Patient 3		Patient 4		P-values Heparin	MA-CDPS	CE-CDPS	HP-CDPS
	dA	dB	dA	dB	dA	dB	dA	dB				
Heparin												
Δ CD62p ⁺ %	45.5	14.3	45.6	15.1	60.3	42.3	34.8	56.4	Reference			
Ratio	4.2	2.2	2.8	3.9	4.3	8.2	2.7	2.8	Reference			
MA-CDPS												
Δ CD62p ⁺ %	74.8	69.9	9.6	17.4	16.7	32.6	51.1	63.6	0.779	Reference		
Ratio	5.2	5.5	1.4	3.5	1.9	8.2	3.5	3.1	0.779	Reference		
CE-CDPS												
Δ CD62p ⁺ %	13.9	14.4	2.4	1.1	7.9	10.2	22.9	45.0	0.017	0.012	Reference	
Ratio	1.8	2.2	1.1	1.2	1.4	2.7	2.2	2.4	0.012	0.012	Reference	
HP-CDPS												
Δ CD62p ⁺ %	67.6	53.0	0.1	1.2	23.3	17.1	63.2	60.7	0.779	0.123	0.025	Reference
Ratio	4.9	5.1	1	1.1	2.1	4.7	4.6	2.8	0.484	0.123	0.036	Reference
CDPS												
Δ CD62p ⁺ %	76.1	73.1	27.5	-2.8	42.7	34.1	63.5	58.1	0.484	0.327	0.017	0.093
Ratio	5.8	6.5	-	0.7	2.5	7	4.4	2.5	0.889	0.889	0.025	0.123

Platelets from two different donors (dA and dB) were incubated with plasma samples containing HIT ab from four patients (Patients 1, 2, 3 and 4) and with the different sulphated polysaccharides. Δ CD62p⁺ %: percentage increases in CD62p⁺ platelets; ratio: %CD62p⁺ (0.3 IU heparin or 5 μ g/ml sulphated polysaccharide)/ %CD62p⁺ (0 IU heparin or 0 μ g/ml sulphated polysaccharide).

synthesis of differently sulphated β -cyclodextrins with different biological activities and toxicological profiles. The introduction of distinct alkyl groups on the β -cyclodextrin glucopyranose unit, for example, did not prolong the aPTT *in vitro*, suggesting a reduced anti-coagulant activity compared with CDPS [28, 29]. These compounds were tested on their potential to improve chondrocyte repair function, on their anti-coagulant properties and on their potential to activate human platelets in an *in vitro* model of HIT.

Both ME-CD-3-S and ME-CD-6-S were unable to affect IL-6 release and aggrecan production by chondrocytes *in vitro*, indicating that monosulphated β -cyclodextrin derivatives were unable to affect cartilage metabolism. The presence of at least two sulphate groups per glucopyranose unit of the β -cyclodextrin ring—irrespective of their position or spacing—was necessary to block down-stream IL-1 events and to warrant an enhancement of the chondrocyte's anabolic function. It was concluded that the introduction of alkyl groups was of minor importance compared with the degree of sulphating, when cartilage protection was considered. Additional experiments confirmed the positive effect of CE-CDPS, mainly, on the chondrocyte's anabolic activity (SOX-9, aggrecan). On the catabolic side, MMP2 expression was slightly decreased upon CE-CDPS stimulation. To provide in-depth insights on the potential anti-catabolic activity of CE-CDPS, future experiments on cell cultures co-stimulated with pro-inflammatory cytokines are warranted.

The *in vitro* tests with MA-CDPS, HP-CDPS, CE-CDPS and CDPS on blood coagulation revealed dose-related anti-coagulant effects but no remarkable mutual

difference between the derivatives. At presumed therapeutic concentrations (5 μ g/ml *in vitro*), none of the polysulphated β -cyclodextrin derivatives revealed an inhibition of the blood coagulation cascade. Moreover, MA-CDPS, HP-CDPS and CDPS showed an interaction with the heparin/PF4 antibodies, triggering activation of the platelets. Introduction of an average of three carboxyethyl chains on the β -cyclodextrin ring, in contrast, significantly decreased platelet activation in comparison with heparin and the other β -CDPS derivatives ($P < 0.05$) in this *in vitro* model of HIT, indicating that CE-CDPS exhibits no cross-reactivity with the heparin/PF4 antibodies.

In vitro testing of the interaction of CDPS derivatives with HIT Ab-positive sera in some way constrain the conclusions of the present study. There are no data available on the ability of the derivatives to induce auto-immune antibodies *in vivo* themselves. A previously published case report on the induction of HIT after chondroitin polysulphate therapy showed antibodies directed against epitopes of chondroitin polysulphate, but HIT only developed after an additional heparin treatment, which suggests cross-reactivity [13]. These data suggest the potential of polysulphated polysaccharides to induce auto-antibodies although these polysaccharides do not invariably induce HIT themselves. Nevertheless, being low-molecular weight polysaccharides, the possible occurrence of HIT following the administration of CDPS *in vivo* may be less probable as classical HIT rarely occurred with low-molecular weight forms of heparin.

In summary, these studies aimed at revealing more advanced polysaccharide polysulphates with chondro-protective capacities. We showed that poly- but not

monosulphated β -cyclodextrins can restore ECM repair potential of OA chondrocytes *in vitro*. From these polysulphated β -cyclodextrin derivatives, CE-CDPS had a better safety profile when the *in vitro* induction of HIT was considered. Additional pre-clinical studies in models of OA are underway to evaluate the *in vivo* chondroprotective efficacy of CE-CDPS.

Rheumatology key messages

- CDPSs restore synthesis of ECM molecules of OA chondrocytes, depending on the sulphation degree.
- Carboxyethyl- β CDPS can restore OA chondrocyte metabolism without enhanced risk of HIT.

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