

# Characterization of human placental glycosaminoglycans and regional binding to VAR2CSA in malaria infected erythrocytes

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**Abstract** Placental malaria is a serious problem in sub-Saharan Africa. Young women are particularly susceptible to contracting this form of malaria during their first or second pregnancy despite previously acquired immunity from past infections. Placental malaria is caused by *Plasmodium falciparum* parasites expressing VAR2CSA on the erythrocyte surface. This protein adheres to a low-sulfated chondroitin sulfate-A found in placental tissue causing great harm to both mother and developing fetus. In rare cases, the localization of infected erythrocytes to the placenta can even result in the vertical transmission of malaria. In an effort to better understand this infection, chondroitin sulfate was isolated from the cotyledon part of the placenta, which should be accessible for parasite adhesion, as well as two non-

accessible parts of the placenta to serve as controls. The placental chondroitin sulfate structures and their VAR2CSA binding were characterized. All portions of human placenta contained sufficient amounts of the appropriate low-sulfated chondroitin sulfate-A to display high-affinity binding to a recombinant truncated VAR2CSA construct, as determined using surface plasmon resonance. The cotyledon is the only placental tissue accessible to parasites in the bloodstream, suggesting it is the primary receptor for parasite infected red blood cells.

**Keywords** Placental malaria · VAR2CSA · Surface plasmon resonance · PfEMP1 · Chondroitin sulfate · Disaccharide analysis

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## Abbreviations

AMAC	2-aminoacridone
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
EDTA	ethylenediaminetetraacetic acid
GAG	glycosaminoglycan
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
pfEMP	<i>Plasmodium falciparum</i> erythrocyte membrane protein
SPR	surface plasmon resonance
VAR2CSA	a PfEMP1 protein

## Introduction

Malaria is a serious health concern, endemic to 140 countries around the world. In 2012, the World Health Organization estimates there were approximately 219 million cases of

malaria, resulting in 660,000 deaths [1]. Malaria is caused by infection by any of five *Plasmodium* species; *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, or *P. knowlesi*. Infection by *P. falciparum* is the most deadly and predominates in Africa, where 90 % of malaria deaths occur [1]. What makes the *P. falciparum* parasite especially virulent is its unique ability to insert proteins, functioning as adhesins, into the membrane of the infected erythrocyte. These adhesins, called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins, bind various receptors in the host microvasculature allowing the infected erythrocytes to sequester and avoid clearance in the spleen [2]. People living in endemic regions acquire protective immunity to malaria as a function of age [3]. Clinical immunity is correlated with the buildup of antibodies capable of inhibiting sequestration, by blocking the interaction between the expressed PfEMP1 proteins and its host receptors [4]. Pregnant women are especially susceptible to infection, despite previously acquired immunity. There are numerous maternal and fetal complications associated with malaria in pregnant women including severe anemia, pulmonary edema, kidney failure, pre-eclampsia, low-birth weight, premature delivery, miscarriage, and death [5–10]. Placental malaria is caused by a subgroup of parasites expressing a distinct PfEMP1 protein, called VAR2CSA, that enables the infected erythrocyte to adhere to chondroitin sulfate-A in the placenta [11–13]. Immunity to placental malaria is developed over successive pregnancies and is correlated with the buildup of anti-VAR2CSA antibodies capable of blocking the VAR2CSA-chondroitin sulfate-A interaction [14, 15]. This supports the use of VAR2CSA in a vaccine against *P. falciparum*.

It has been shown that infected erythrocytes selected for placental binding adhere preferentially to a low sulfated form of chondroitin sulfate-A (chondroitin-4-sulfate) in the intervillous spaces of the placental [16]. This region of the placenta is found in the cotyledons of the decidua basalis or the maternal side of the organ [17]. It has further been suggested that the minimal structural requirement within this type of chondroitin sulfate-A is a dodecasaccharide sequence containing two to three 4-*O*-sulfated disaccharide units [18]. VAR2CSA is a large protein (350 kDa) consisting of six Duffy binding-like domains (three DBLX followed by three DBL

domains), a cysteine-rich inter-domain region (termed CIDR<sub>PAM</sub>) between DBL2X and DBL3X, and a number of interdomains [13, 19]. The minimal chondroitin sulfate-A binding region in VAR2CSA is the ID1-DBL2x region of the protein with an additional 93 amino acids on the C-terminus [13, 20, 21].

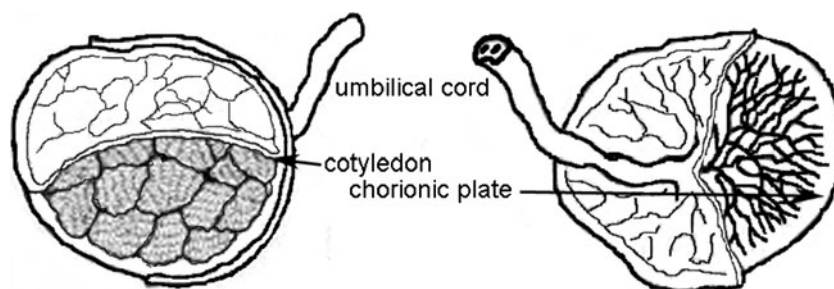
In this study we isolated the GAGs from the placenta cotyledon, chorionic plate (barrier joining maternal and fetal organ portions), and umbilical cord (Fig. 1) for characterization. Chondroitin sulfates isolated from these tissues were characterized by disaccharide analysis and then used for surface plasmon resonance analysis to measure the binding to a VAR2CSA construct including the minimal chondroitin sulfate-A binding domain.

## Materials and methods

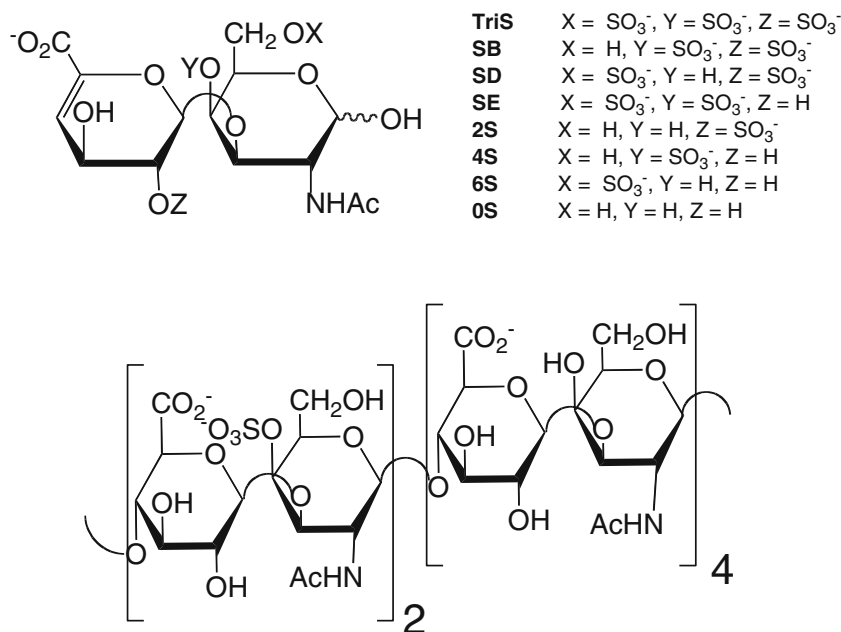
### Materials

Three whole human placentas with umbilical cord still attached were obtained from Cardinal Biologicals (Tyler, Texas). Actinase E was from Kaken Biochemicals (Tokyo, Japan). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and urea were from Sigma Chemical Company (St. Louis, MO). Vivapure Q Mini H columns were from Sartorius Stedium Biotech (Goettingen, Germany). Amicon ultracentrifugal filters (3000 molecular weight cut-off) and 0.22  $\mu$ m stericup filters were from Millipore (Billerica, MA). Unsaturated disaccharides standards of chondroitin sulfate (0S,  $\Delta$ UA-GalNAc; 4S,  $\Delta$ UA-GalNAc4S; 6S,  $\Delta$ UAGalNAc6S; 2S,  $\Delta$ UA2S-GalNAc; 2S4S or SB,  $\Delta$ UA2SGalNAc4S; 2S6S or SD,  $\Delta$ UA2S-GalNAc6S; 4S6S or SE,  $\Delta$ UA-GalNAc4S6S; and TriS,  $\Delta$ UA2SGalNAc4S6S) (Fig. 2), and Chondroitin lyases ABC and ACII were from Associates of Cape Cod, Inc. (East Falmouth, MA). Unsaturated disaccharides standards of heparan sulfate (0S,  $\Delta$ UA-GlcNAc; NS,  $\Delta$ UA-GlcNS; 6S,  $\Delta$ UA-GlcNAc6S; 2S,  $\Delta$ UA2S-GlcNAc; 2SNS,  $\Delta$ UA2S-GlcNS; NS6S,  $\Delta$ UA-GlcNS6S; 2S6S,  $\Delta$ UA2SGlcNAc6S; and TriS,  $\Delta$ UA2S-GlcNS6S) were purchased from Seikagaku Corporation (Japan). Expression and purification of the

**Fig. 1** Drawing of human placenta with sections analyzed labeled as umbilical cord, chorionic plate and cotyledon



**Fig. 2** Common chondroitin disaccharides formed through chondroitin lyase treatment (*top*) and chondroitin sulfate undersulfated dodecasaccharide sequence (*bottom*) proposed to bind to infected erythrocytes [18]



recombinant heparin lyase I, II, and III from *Flavobacterium heparinum* were performed using previously established protocols [22–24]. All other chemicals were of reagent grade.

#### Extraction of glycosaminoglycans from placenta tissue

Tissue samples were stored at 4 °C until free of ice. Excess blood was washed from the tissue using chilled phosphate buffered saline. The whole placenta was dissected based on the three regions of the organ present, the cotyledon, the chorionic plate, and the umbilical cord [17].

Samples from each region were lyophilized. Completely dry samples were ground into a fine powder. Tissue was defatted using a series of chloroform and methanol washes using 2:1, 1:1, 1:2 v/v ratios. Washes were carried out overnight using a stir-plate in a fume hood. The defatted sample was resuspended using the minimal volume of water and proteolyzed using 1 % actinase E (20 mg/mL) at 55 °C. After proteolysis, dry urea and CHAPS were combined with the mixture to form a solution of 8 M urea and 2 wt% CHAPS. After the mixture equilibrated it was centrifuged to remove solid residue. The supernatant was then passed through a 0.22 μm filter. GAGs were isolated using Vivapure Q Maxi H spin columns. Columns were equilibrated with 3 mL 8 M urea with 2 % CHAPS. Samples were loaded onto the column at 500 × g and then washed, first with 5 mL 8 M urea with 2 % CHAPS, then five-times with 5 mL 200 mM NaCl. GAGs were then released from the spin columns by washing three-times with 1 mL 16 % NaCl. Using an 80 vol% methanol, the GAGs were precipitated from the 16 % NaCl solution. The white precipitate was recovered using centrifugation and resuspended in 1 mL water for further analysis. The amount of

GAG isolated from each region was determined using a carbazole assay [25].

#### Isolation of chondroitin sulfate for gel permeation chromatography and SPR analysis

Intact chondroitin sulfate samples were isolated from the whole GAG samples by subjecting the sample to 10 mU of heparin lyases I, II, and III for 10 h at 37 °C [26]. Heparin lyases were then denatured by placing samples in 100 °C water bath for 10 min. Samples were centrifuged at 10,000 × g for 5 min to pellet denatured enzyme. Supernatant was purified using 3000 molecular weight cut-off spin column. Retentate contained intact chondroitin sulfate, which was lyophilized and kept for molecular weight analysis and for use in SPR analysis and disaccharide analysis. Permeate, containing heparan sulfate disaccharides, was kept for use in disaccharide analysis.

#### Disaccharide analysis by ultraperformance liquid chromatography-mass spectrometry

Chondroitin sulfate disaccharides were prepared by depolymerizing each chondroitin sulfate sample with chondroitinases ABC and ACII [26]. Heparan sulfate disaccharides, chondroitin sulfate disaccharides and heparan sulfate disaccharide standards and chondroitin sulfate disaccharide standards were labeled with 2-aminoacridone (AMAC) [27] and analyzed by liquid chromatography-mass spectrometry using a previously described method [26].

**Table 1** Average calculated chondroitin sulfate molecular weight of the placenta by region

Sample	M <sub>N</sub> (kDa)	M <sub>W</sub> (kDa)	Polydispersity
Cotyledon	45.2±12.9	49.1±13.8	1.09±0.07
Chorionic Plate	41.7±5.2	45.3±7.7	1.11±0.04
Umbilical Cord	46.7±3.4	54.0±2.8	1.16±0.02
Overall Average	44.1±6.9	49.4±7.6	1.13±0.06

Average molecular weight calculation using gel permeation high performance liquid chromatography

Gel permeation high performance liquid chromatography was performed on the intact chondroitin sulfate samples using a 30 cm×7.8 mm TSK-GEL G4000PWxl size exclusion column, a Shimadzu LC-10Ai pump, Shimadzu CBM-20A controller, and a Shimadzu RID-10A refractive index detector. The mobile phase was 0.01 M NaNO<sub>3</sub> at a flow rate of 0.6 mL/min. The column was maintained at 40 °C and the sample volume injected was 20 µL (5 mg/mL). A calibration curve was created using 30.6 kDa, 43.8 kDa, 78.7 kDa, 130.2 kDa hyaluronan molecular weight markers (Hyalose LLC, Oklahoma City, OK). All gel permeation high performance liquid chromatography chromatograms were recorded with LC Solution Version 1.25 software and analyzed with the “GPC Postrun” function [28].

Preparation of the chondroitin sulfate sensor chip

BIAcore 3000 (GE Healthcare, Uppsala, Sweden) was used to measure the interaction between the DBL3x region of VAR2CSA and chondroitin sulfate isolate from the cotyledon, chorionic plate, and umbilical cord. These regions were chosen based on their expected proximity to iRBCs. Chondroitin sulfates were isolated from different regions of the placenta

from which heparan sulfate had been removed using heparinases, were biotinylated with NHS-PEG<sub>4</sub>-biotin (Thermo Scientific, Rockford, IL). Dry chondroitin sulfate samples were dissolved in PBS, pH 7.2 and combined with 20-mmol excess of fresh NHS-PEG<sub>4</sub>-Biotin in ultrapure water. Incubation of the reaction for 30 min at room temperature was followed by purification with 3000 molecular weight cut-off spin columns and lyophilization. Biotinylated chondroitin sulfate samples were immobilized on strepavidin chip using the manufacturer’s protocol. Briefly, 20 µL of biotinylated chondroitin sulfate sample from the cotyledon, chorionic plate, and umbilical cord dissolved in HBS-EP (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM sodium chloride, 3 nM ethylenediaminetetraacetic acid (EDTA), 0.005 % polysorbate P20, pH 7.4) running buffer was injected over flow cell 2, flow cell 3, and flow cell 4, respectively, of the streptavidin chip at a flow rate of 10 µL/min. Successful immobilization was indicated by a change in resonance units greater than or equal to 100 resonance units after injection. Flow cell 1 was prepared as a control cell by a 1 min injection of a saturated solution of biotin.

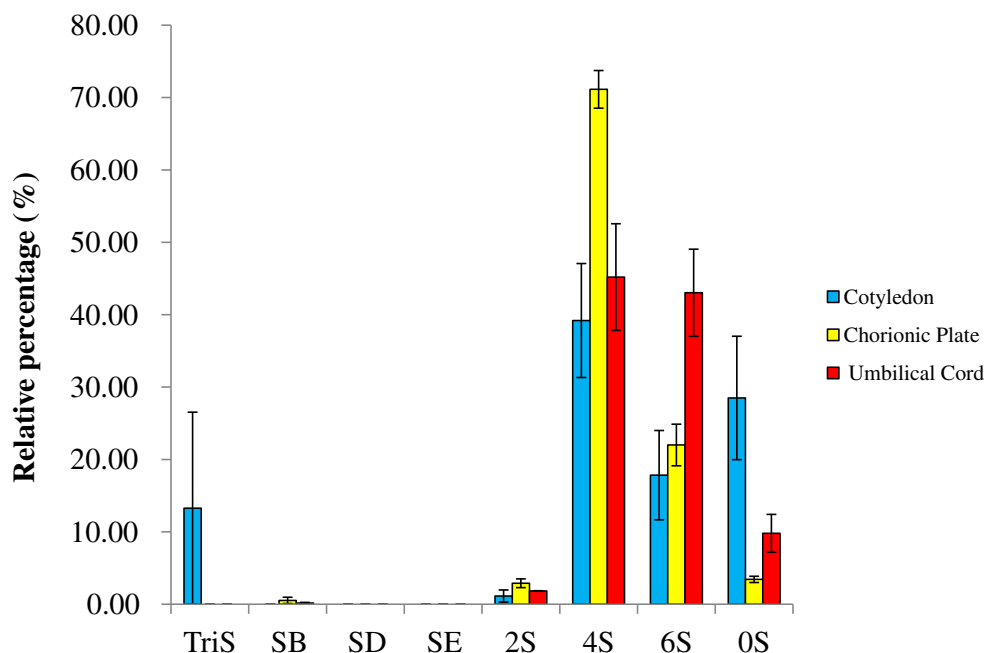
Measurement of interaction between VAR2CSA and regional placental chondroitin sulfate

The MP907 construct of VAR2CSA, prepared by a method previously described by Clausen and Salanti [13], is a construct that contains the minimal chondroitin sulfate-A binding region. The protein construct was diluted in HBS-EP running buffer to 250 nM, 125 nM, 63 nM, 32 nM, and 16 nM. Each dilution was injected (90 µL) at a flow rate of 30 µL/min, followed by a 3 min dissociation period of running buffer flowing over the sensor. To ensure complete regeneration, 30 µL of 2 M NaCl was injected before the next sample injection.

**Table 2** Total relative disaccharide composition of three placental samples 1, 2, and 3 from various regions of the placenta

Samples	Heparan sulfate disaccharides								Chondroitin sulfate disaccharides								OS <sub>HA</sub>	Ratio of chondroitin sulfate to heparan sulfate
	Tri S	NS6S	NS2S	NS	2S6S	6S	2S	OS	Tri S	SB	SD	SE	2S	4S	6S	OS		
1 Cotyledon	0.2	0.4	0.5	5.1	–	0.3	1.3	14.3	18.2	–	–	–	0.2	12.7	2.9	11.7	32.1	5:1
1 Chorionic plate	0.1	0.3	0.7	3.7	–	0.3	0.8	3.4	–	0.2	–	–	2.0	55.1	22.8	2.8	7.8	9:1
1 Umbilical Cord	–	–	0.1	0.5	–	0.1	1.0	0.5	–	0.3	–	–	1.8	56.9	29.8	7.4	1.5	44:1
2 Cotyledon	0.1	0.2	0.3	3.9	–	0.2	0.1	7.7	–	–	–	–	0.1	19.0	10.6	23.8	33.9	4:1
2 Chorionic plate	0.2	0.2	0.4	2.8	–	0.2	0.5	2.5	–	1.3	–	–	2.1	67.5	19.6	4.0	3.4	13:1
2 Umbilical Cord	–	–	0.2	1.2	–	0.1	1.4	0.9	–	0.2	–	–	1.5	36.2	41.5	5.7	7.4	23:1
3 Cotyledon	0.4	0.3	0.5	3.7	0.4	–	0.6	14.0	–	–	–	–	1.7	32.7	16.5	9.3	19.8	3:1
3 Chorionic Plate	0.1	0.1	0.2	1.1	0.2	–	0.5	1.8	–	–	–	–	3.8	70.8	16.7	2.5	2.0	24:1
3 Umbilical Cord	–	–	0.3	1.1	0.1	–	1.2	1.4	–	–	–	–	1.6	29.7	43.1	13.1	8.9	21:1

**Fig. 3** Normalized average chondroitin sulfate disaccharide composition based on data in Table 2. Standard deviation represents variation between placenta samples



## Results and discussion

### GAG isolation and chondroitin sulfate purification from three regions of placenta

To isolate GAGs from three regions of placenta tissues (cotyledon, chorionic plate, and umbilical cord), we used a simple four-step procedure involving: 1. Defatting; 2. protease digestion; 3. strong-anion-exchange chromatography on a spin column; and 4. methanol precipitation. This procedure had been previously established in our laboratory for the quantitative isolation of heparin from human plasma [29]. The total GAG isolated from each sample was subject to structural and biological activity analysis. Chondroitin sulfate from each sample was further purified from the total GAG by selective polysaccharide digestion using heparin lyases.

### Calculated molecular weights of isolated chondroitin sulfate

GPC analysis of chondroitin sulfate indicates average molecular weights from 36 kDa to over 60 kDa (Table 1). The

average weighted molecular weight of the samples collected was 49.4 kDa. Chondroitin sulfate, especially that associated with blood vessels, can range between 20–80 kDa [30, 31]. A previous placental glycosaminoglycan recorded average relative molecular weight ( $M_r$ ) of GAGs isolated to range from 46 kDa to 60 kDa [16].

### Disaccharide analysis

Ultraperformance liquid chromatography-mass spectrometric analysis of the resulting disaccharides was carried out using the AMAC-conjugated protocol to examine all 17 potential disaccharides represented by the standards. Disaccharide analysis affords the relative amounts of each disaccharide and also allows us to calculate the ratio of heparan sulfate disaccharides to chondroitin sulfate disaccharides (Table 2). Chondroitin sulfate is the dominant GAG component in all three umbilical cord samples with chondroitin sulfate to heparan sulfate ratios of 44:1, 23:1, and 21:1. Cotyledons exhibit the lowest ratios (5:1, 4:1, and 3:1) while the chorionic plate varied considerably from sample to sample (9:1, 13:1, and 24:1). The analysis

**Table 3** Summary of kinetic data of regional isolated chondroitin sulfate with MP907 VAR2CSA construct

Interaction	$k_{on}$ ( $M^{-1} s^{-1}$ )	$k_{off}$ ( $s^{-1}$ )	$K_D$ (M)
Cotyledon CS-MP907	$3.5 \times 10^4 (\pm 1.6 \times 10^3)$	$1.3 \times 10^{-3} (\pm 6.2 \times 10^{-5})$	$3.7 \times 10^{-8}$
Chorionic Plate CS-MP907	$7.1 \times 10^4 (\pm 1.1 \times 10^3)$	$1.1 \times 10^{-3} (\pm 2.4 \times 10^{-5})$	$1.6 \times 10^{-8}$
Umbilical Cord CS-MP907	$8.2 \times 10^4 (\pm 1.1 \times 10^3)$	$1.4 \times 10^{-3} (\pm 2.9 \times 10^{-5})$	$1.7 \times 10^{-8}$

( $\pm$  standard errors were from the global fitting of different protein concentrations)



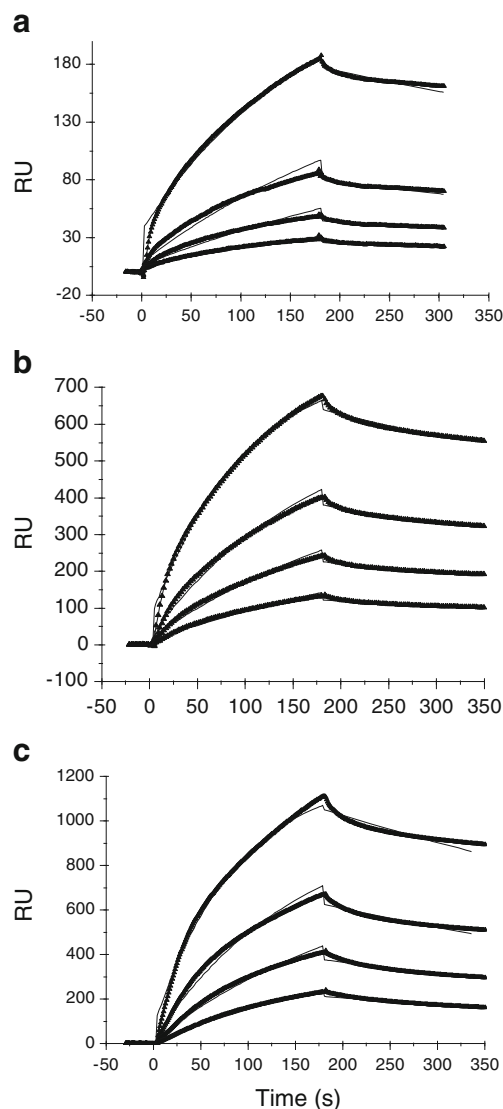
becomes more meaningful when examining the normalized average chondroitin sulfate composition based on regions of the placenta (Fig. 3). From this perspective the low-sulfation content of placental chondroitin sulfate is apparent. Over two-thirds of chondroitin sulfate found in the chorionic plate is chondroitin sulfate-A, the major form recognized substrate of VAR2CSA. The umbilical cord contains equivalent fractions of CS4S and CS6S. Meanwhile the cotyledons are composed of a lower percentage of CS4S but a much higher percentage of CS0S. Chondroitin 4-*O*-sulfate and unsulfated chondroitin are the two most recognized substrates for erythrocyte membrane protein binding. Based on these populations we would predict that cotyledon chondroitin sulfate should show the strongest binding to erythrocyte membrane protein.

#### Surface plasmon resonance (SPR) analysis

SPR binding analysis was carried out to measure the interaction between the isolated placenta chondroitin sulfate samples and VAR2CSA (MP907). Different concentrations of MP907 were injected over a sensor chip containing cotyledon, chorionic plate, and umbilical cord chondroitin sulfate. Sensorgrams were processed using Biaevaluation 4.0.1 software (Fig. 4). Kinetics and binding affinity (on/off-rates and dissociation constants) was calculated by applying 1:1 Langmuir binding in accordance with past experiments [13, 32]. Based on the binding kinetic data (Table 3), the chondroitin sulfate from chorionic plate, and umbilical cord showed slightly higher affinity (lower  $K_D$ ) to VAR2CSA protein than the chondroitin sulfate from cotyledon.

#### Conclusions

Our results confirmed findings of many other studies on placental chondroitin sulfate interaction with *P. falciparum* infected erythrocytes [13, 16, 18]. Differences in characterization are associated with differences in analytical method and detection sensitivity. In all analyses, cotyledon chondroitin sulfate displays the presence of predominantly 4S and 0S disaccharides. The observation of 6S disaccharide and other minor disaccharides in the current study may be attributable to the inclusion of GAGs from the fibrous scaffold of the lobes and/or the increased sensitivity of the ultraperformance liquid chromatography-mass spectrometric method used. The other three regions examined also had substantial fractions of 4S disaccharides. However they did not possess the same level of unsulfated disaccharide. The impact of this difference was next assessed using SPR. Cotyledon, chorionic plate, and umbilical cord GAGs all initiated a binding of the VAR2CSA construct MP907. All three placental tissues showed similar tight binding, with only a ~2-fold range in binding affinities. Despite slightly weaker binding to the cotyledon chondroitin



**Fig. 4** SPR sensorgrams of isolated chondroitin sulfate (from different regions of placenta) and MP907 interaction. **a**: Cotyledon; **b**: Chorionic Plate; **c**: Umbilical Cord. Concentrations of MP907 (from top to bottom): 125, 63, 32, and 16 nM, respectively. The black curves are the fitting curves. SPR experiments relied on duplicate injections performed after the regeneration of the chip surface. Standard errors were calculated from the global fitting of different protein concentrations and are presented in Table 2

sulfate, the maternal blood flow with the circulating infected erythrocytes only have access to this part of the placenta.

Future work will focus on the prevention or treatment of malaria. There are several possible approaches. It is established that once a mother produces antibodies preventing VAR2CSA binding the symptoms of placental malaria are significantly reduced resulting in healthier, heavier neonates than those born to infected mothers without antibodies [12, 33]. This suggest that one of the several conserved domains of VAR2CSA could be a potential vaccine candidate. Since VAR2CSA is a very large protein, current opinion is to use a construct of VAR2CSA that contains the minimal strong

chondroitin sulfate-A binding domain [13]. This region enables the strongest binding of erythrocytes and therefore is more desirable to block infected erythrocytes from the endothelial membrane.

Another treatment approach focuses on the GAG to keep infected erythrocytes in circulation resulting in their destruction by the spleen. A chondroitin sulfate-A oligosaccharide or polysaccharide might be used to bind to infected erythrocytes so that they cannot adhere to endothelium. Such a therapeutic target must be a stronger binder than the low-sulfated placental chondroitin sulfate A for this approach to be effective and must also stay in the circulation long enough to go through several blood-phase reproduction cycles. One advantage of using a GAG therapeutic is that many anti-malarials can produce intense side effects and GAGs generally are better tolerated. The development of a therapeutic chondroitin sulfate-oligomer would require more extensive kinetic studies.

Although malaria and other mosquito-borne illnesses are a serious global health concern, the areas that bear the brunt of mortalities are severely impoverished. Malaria treatment is not economically attractive option for drug companies. The information gathered from characterization of substrates and ligand kinetics will be integral in developing cost-effective treatments to lessen the severity or eliminate placental malaria.

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