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Visualization of Nanofibrillar Cellulose in Biological Tissues Using a Biotinylated Carbohydrate Binding Module of β-1,4-Glycanase

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ABSTRACT: Nanofibrillar cellulose is a very promising innovation with diverse potential applications including high quality paper, coatings, and drug delivery carriers. The production of nanofibrillar cellulose on an industrial scale may lead to increased exposure to nanofibrillar cellulose both in the working environment and the general environment. Assessment of the potential health effects following exposure to nanofibrillar cellulose is therefore required. However, as nanofibrillar cellulose primarily consists of glucose moieties, detection of nanofibrillar cellulose in biological tissues is difficult. We have developed a simple and robust method for specific and sensitive detection of cellulose fibers, including nanofibrillar cellulose, in biological tissue, using a biotinylated carbohydrate binding module (CBM) of β-1,4-glycanase (EXG:CBM) from the bacterium Cellulomonas fimi. EXG:CBM was expressed in Eschericia coli, purified, and biotinylated. EXG:CBM was shown to bind quantitatively to different nanofibrillar celluloses. Biotinylated EXG:CBM was used to visualize cellulose fibers by either fluorescence- or horse radish peroxidase (HRP)-tagged avidin labeling. The HRP-EXG:CBM complex was used to visualize cellulose fibers in both cryopreserved and paraffin embedded lung tissue from mice dosed by pharyngeal aspiration with 10–200 μg/mouse. Detection was shown to be highly specific, and the assay appeared very robust. The present method represents a novel concept for the design of simple, robust, and highly specific detection methods for the detection of nanomaterials, which are otherwise difficult to visualize.

INTRODUCTION

Nanofibrillar cellulose (also called nanocellulose) is a dense network of highly fibrillated celluloses ranging from 10 to 100 nm in diameter and several micrometers in length.1,2 Nanofibrillar cellulose constitutes an innovation potential within the forest sector since nanofibrillar cellulose can be used as high strength materials in products such as high quality paper, coatings, cosmetics, vehicles, furniture, food, electronic components, and pharmaceuticals. Recently, the production of nanofibrillar cellulose on an industrial scale has become economically feasible.1,3,4 Because of their mechanical properties and low production costs, nanofibrillar cellulose constitutes an attractive alternative to other nanofibers, e.g., carbon nanotubes.4,5 Nanofibrillar cellulose is produced by the mechanical refining of wood or natural fiber pulp, followed by either grinding or homogenization.2

Workers exposed to cotton dust, which principally consists of cellulose, have been reported to develop chest tightness, hyperresponsiveness, and chronic bronchitis.6 Exposure to wood dust (where cellulose is the major constituent) causes cancer of the nose and the paranasal sinuses,7 and exposure to textile fibers in weavers has also been considered possibly carcinogenic.6,7 Cellulose fibers have been shown to persist in the lungs of rats with a half-life in the range of 1000 days after intratracheal deposition.8 Workers may be exposed to airborne nanofibrillar cellulose particles from grinding or spray drying during production.1 However, data on health effects in workers exposed to nanofibrillar cellulose are at present largely unavailable.1 There are reasons for health concern related to...
nanofibrillar cellulose since they, like asbestos and carbon nanotubes, are high aspect ratio materials, and exposure could cause physiological responses (e.g., inflammation) in the respiratory tract. Studies of nanomaterial toxicity are often hampered by difficulties in detecting and quantifying the nanomaterial in biological tissues. This is especially true for carbon-based nanomaterials like nanofibrillar cellulose, which mainly consists of glucose moieties.

Exoglucanases (EXG) bind cellulose with very high specificity and affinity via the cellulose binding domain EXG:CBM. 10 We have utilized the specific binding properties of EXG:CBM to develop a new detection method for the visualization of cellulose. Here, we show that biotinylated EXG:CBM can be used to visualize pulp cellulose as well as four different types of nanofibrillar cellulose fibers in biological tissue, that biotinylation of EXG:CBM improves the binding properties of EXG:CBM to cellulose, and that specific staining can be obtained using both fluorescence- and horse radish peroxidase (HRP)-tagged avidin labeling.

### METHODS

#### Production and Purification of EXG

The plasmid pJexpress401:22788 carries the coding sequence corresponding to the 110 amino acid long EXG:CBM [PDB: 1EXG] from the gene Cex (β-1,4-glycanase from the bacterium Cellulomonas fimi). The structure of the EXG:CBM protein has been solved by NMR. 11 EXG:CBM contains a disulfide bond, and to ensure correct disulfide bond formation, the PelB signal peptide sequence from Pectobacterium carotovorum was fused to the reading frame encoding EXG:CBM in order to target EXG:CBM to the periplasm. The reading frame, including the signal peptide, was custom synthesized by DNA2.0 (Menlo Park, CA, USA) in a vector, pJexpress401 (DNA2.0), under control of the T5 promoter and with kanamycin resistance as the selection marker. The codon bias of the EXG:CBM reading frame was optimized for expression in Escherichia coli according to the algorithms of DNA2.0. EXG:CBM was produced from the plasmid pJexpress401:22788 in E. coli strain MAS72 (rec+ derivative of MAS90). 12 MAS72 was grown at 37 °C in buffered rich medium supplemented with salts 13 and 30 μg/mL kanamycin. EXG:CBM synthesis was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the growth media to a final concentration of 0.1 mM at a cell culture density of OD600 = 1, and the cultures were left to grow for 20 h at 37 °C. Cells were harvested, washed, and resuspended in 50 mM Tris-HCl and 1 mM EDTA at pH 7.0. A sequence of 20 s sonication at 40% maximum energy (an amplitude of 8 µm) on a Soniprep 150 (Sanyo) sonicator, followed by a 60 s pause while kept on ice released the protein from the periplasm into the supernatant. Cell debris was removed by centrifugation at 20,000g for 20 min at 4 °C. Protein yields of EXG:CBM from 1 L culture, grown as above, were typically 15–20 mg.

EXG:CBM was purified taking advantage of the affinity of the protein for cellulose 15 by incubating the cell lysate with 1 g (dry weight) of Avicel (Sigma-Aldrich) per liter original culture with agitation for 1 h at 25 °C. The Avicel with bound EXG:CBM was pelleted by centrifugation at 5,000g for 20 min at room temperature, and the supernatant was discarded.

The Avicel pellet was packed in a Pharmacia XK 16 column with a thermostatic jacket and washed with three column volumes of 1 M NaCl followed by the same amount of water. To elute the protein, the temperature of the column was ramped up from 70 to 82 °C during 20 min, while continuously running water over the column (2 mL/min). EXG:CBM was eluted, and fractions containing protein (as determined by absorbance at 280 nm) were collected and lyophilized. After dissolving in water, insoluble material was pelleted by centrifugation as described above. The yield of EXG:CBM at this point was typically 80–90%, and the protein was more than 95% pure as judged by SDS–PAGE (Figure 1A, lane 3). However, when performing assays...
Table 2. Binding Data for EXG to the Different Types of Cellulose

<table>
<thead>
<tr>
<th>Cellulose Type</th>
<th>$K_D$ (μM)</th>
<th>$B_{max1}$</th>
<th>$K_D$</th>
<th>$B_{max2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF2</td>
<td>0.0063 ± 0.0023</td>
<td>1.46 ± 0.17</td>
<td>0.65 ± 0.28</td>
<td>2.47 ± 0.28</td>
</tr>
<tr>
<td>CF1</td>
<td>0.010 ± 0.0055</td>
<td>1.33 ± 0.22</td>
<td>0.72 ± 0.31</td>
<td>2.03 ± 0.19</td>
</tr>
<tr>
<td>CF3</td>
<td>0.022 ± 0.0062</td>
<td>2.28 ± 0.35</td>
<td>0.76 ± 0.56</td>
<td>2.36 ± 0.37</td>
</tr>
<tr>
<td>CF4</td>
<td>0.055 ± 0.0083</td>
<td>2.69 ± 0.09</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nylon filter</td>
<td>0.084 ± 0.017</td>
<td>3.24 ± 0.17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CF1-CF4</td>
<td>0.35 ± 0.054</td>
<td>1.07 ± 0.038</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Binding constants ($K_D$) and maximum binding ($B_{max}$) and (-) indicate that only a one-site binding model was used for fitting. Data were fitted to a two-site binding model, when this was appropriate.

Analysis of Binding Data. Results from binding experiments were analyzed using the OriginLab 9.1 software by fitting the data obtained from equations for one-site binding or two-site binding isotherms to the data:

\[
\text{one-site binding: } [\text{bound}] = \frac{B_{max}[L]}{K_D + [L]} \\
\text{two-site binding: } [\text{bound}] = \frac{B_{max1}[L]}{K_D1 + [L]} + \frac{B_{max2}[L]}{K_D2 + [L]}
\]

where \([\text{bound}]\) is the concentration of EXG:CBM bound to the cellulose and removed from the sample during centrifugation. $B_{max}$ is the calculated concentration of binding sites in the sample. $K_D$ designates the free EXG:CBM concentration obtained in the sample that passed through the spin filter (see above procedure). The data analysis was performed after subtracting the binding of EXG:CBM binding to the nylon membrane of the spin-filters.

Celluloses. Four nanofibrillar cellulose materials (denoted CF1-CF4) and one bulk-sized cellulose fiber (denoted CFS) were used in the study. All cellulose samples were derived from natural wood-based pulp, and they consisted of glucose, xylose, and mannose. Material characteristics are presented in Table 1. The materials were produced and provided by Stora Enso Oyj and UPM Kymmene Oyj. Physicochemical characteristics of the cellulose fibers were provided by the manufacturers. Fiber length was determined by scanning electron microscopy (Zeiss Model EVO MA 10) and laser diffraction (Horiba LA 950). Fiber width was determined by atomic force microscopy (Cypher System, Asylum Research). Zeta potential was determined by dynamic light scattering (Malvern Zetasizer Nano).

Dispersion of Celluloses. Cellulose dispersions were prepared in phosphate buffered saline (PBS, Lonza, Basel, Switzerland). The amount of cellulose needed was either measured with a 1 mL syringe (SOFT-JECT Insulin U-100) or weighed due to high viscosity of the material. Cellulose was dispersed in PBS by high speed vortexing for 10 min to yield a 4 mg/mL stock dispersion. Further dilutions were prepared from the stock dispersion by a dilution series, followed by vortexing for 10 min. Immediately before administration, the dispersions were vortexed quickly.

Animals. Female C57BL/6 mice were obtained from Scanbur AB (Karlslund, Denmark) and quarantined for 1 week. The mice were 7–8 weeks old at arrival and were housed in groups of 4 mice per cage in humidity- and temperature-controlled ventilated rooms with a 12 h day/night cycle. Rodent diet (Altromin no. 1314 FORTI, Altromin Spezialfutter GmbH & Co., Germany) and water were provided ad libitum. Cage-side clinical observations were conducted on a daily basis during the study.

Mice in groups of 8 were exposed to 10, 40, 80, or 200 μg of each of CF material in a 50 μL volume (corresponding to 0.2, 0.8, 1.6, or 4 mg/mL dispersions) by single pharyngeal aspiration. Since PBS was used as vehicle, mice in the control group were exposed to 50 μL of PBS. The mice were sacrificed by an overdose of isoflurane 24 h after exposure. A slice from the left lung was fixed in formalin, while another slice from the left lung was embedded in O.C.T. compound (optimal cutting temperature) (TissueTek, Sakura, The Netherlands) and snap frozen on dry ice.
The study was approved by the Animal Experiment Board and the State Provincial Office of Southern Finland.

**Histology and Immunohistochemistry.** The formalin fixed samples were trimmed, dehydrated, and embedded in paraffin. Sections from both formalin fixed and frozen tissues were cut at 3 μm on a Microtome (Leica Microsystems, Wetzlar, Germany). For general morphology, the sections were stained with hematoxylin and eosin (H&E) stain.

**Figure 2.** Specific staining of CF1 with EXG:CBM in paraffin embedded lung tissue sections from mice exposed to CF1. (A) H&E staining of paraffin embedded lung tissue from mice exposed to 200 μg of CF1. Microscope objective ×10. Arrows locate unstained cellulose fibers. (B) Serial section of the same tissue as in panel A stained with biotinylated EXG:CBM conjugated to HRP, counterstained with Mayer’s hematoxylin. Arrows locate cellulose fibers identified by the EXG:CBM staining.

**Figure 3.** Specific staining of CF1 with biotinylated EXG:CBM in paraffin embedded and cryopreserved lung tissues, counterstained with Mayer’s hematoxylin. (A) Cryopreserved lung sections from mice exposed to 200 μg of CF1 stained with biotinylated EXG:CBM. Microscope objective 10×. Arrows locate cellulose fibers identified by the EXG:CBM staining. (B) Paraffin embedded tissue from vehicle (PBS) exposed mice stained with biotinylated EXG:CBM. Microscope objective 10×. Arrows locate intrabronchially located cellulose fibers identified by the EXG:CBM staining. (D) Control staining of a serial section of the same tissue as that in panel C stained using the immunohistochemistry protocol but excluding biotinylated EXG. Arrows locate unstained cellulose fibers. Microscope objective is 10×.
For EXG:CBM staining, frozen sections were fixed in acetone for 5 min, or paraffin was removed from the sections, and the sections were washed with PBS. Endogenous peroxidase was blocked by Ultravision Hydrogen Peroxide Block (Thermo Scientific, Fremont CA, USA) for 10 min. After washing with PBS, 30% rabbit normal serum with avidin from Avidin/Biotin Blocking kit, Vector Laboratories (Burlingame, CA, USA) was applied to the sections for 30 min and then removed by gentle suction. Biotinylated EXG (final dilution 1:300 with biotin from Avidin/Biotin Blocking kit) was applied to the sections for 60 min. After washing with PBS, Streptavidin Peroxidase Conjugated (Rockland, Limerick, PA, USA) was applied to the sections for 30 min at a dilution of 1:300. After washing in PBS, peroxidase enzyme activity was visualized by incubation in Large Volume AEC Chromogen Single Solution (Thermo Scientific) for 5 min. The sections were then counterstained with Mayer’s hematoxylin, dried and mounted.

Fluorescence Staining for Cellulose. For fluorescence staining, tissue sections (from paraffin or OCT) were blocked with avidin from Avidin/Biotin Blocking kit, Vector Laboratories (Burlingame, CA, USA) in 2% rat serum in nanopure water for 10 min. The slides were then blocked with biotin from Avidin/Biotin Blocking kit for 10 min. Then, biotinylated EXG (diluted 1:300 final concentration) was applied to the sections for 60 min. After washing in PBS, avidin FITC conjugate (ImmunoPure Avidin, Fluorescein Conjugated, Thermo Scientific) was added to the slides for 30 min in the dark. After washing, the slides were mounted and analyzed.

RESULTS

Protein labeling. EXG:CBM has two potential biotinylation sites, the N-terminus and the ε-amine of lysine. Successful biotin-labeling was indicated by the increased masses of EXG:CBM of 226 or 452 Da, respectively, detected by MALDI-TOF MS, indicating single and double biotinylation along with a small signal from unlabeled EXG:CBM (Figure 1B).

Celluloses. Four nanofibrillar cellulose materials (denoted CF1–CF4) and one bulk-sized cellulose fiber (denoted CF5) were used in the study (Table 1). EXG:CBM Binding to Nanofibrillar Cellulose. Four different types of nanofibrillar cellulose and one bulk cellulose were deposited into the lungs of mice by aspiration, and the mice were sacrificed after 24 h. Biotinylated EXG:CBM bound to avidin-HRP was used to visualize cellulose fibers in lung tissue. CF1 was faintly visualized in lung tissue by H&E

Figure 4. HRP-EXG:CBM staining of lung sections from mice dosed with different amounts of CF1. Paraffin embedded lung sections were stained with biotinylated EXG:CBM and counterstained with Mayer’s hematoxylin. Microscope objective is 20×. A, 200 μg of CF1; B, 80 μg of CF1; C, 40 μg of CF1; and D, 10 μg of CF1. Arrows locate cellulose fibers identified by the EXG:CBM staining.
staining (Figure 2A) and clearly visualized following incubation with biotinylated EXG:CBM followed by avidin conjugated to HRP (Figure 2B). The HRP staining appeared as dark red spots in the alveolar sacs/ducts. EXG-HRP staining of CF1 was also demonstrated on cryopreserved lung tissue from mice dosed with CF1 by aspiration (Figure 3A). Specificity was demonstrated by the lack of HRP staining in EXG:CBM-stained lung tissue from the vehicle-exposed mice (Figure 3B) and by the lack of HRP staining when CF1-exposed lung tissue was stained with avidin conjugated to HRP in the absence of biotinylated EXG:CBM (Figure 3D), versus staining with the presence of EXG:CBM from the same series of sections (Figure 3C), thus confirming that there was no nonspecific binding of HRP-avidin.

Mice were exposed to 200, 80, 40, and 10 μg of CF1 (Figure 4A−D) to assess the sensitivity of the HRP-EXG:CBM staining visualized in lung sections. The five different CF materials were all visualized in lung tissue by EXG:CBM-HRP staining. Thus, CF2 (Figure 5A), CF3 (Figure 5B), CF4 (Figure 5C), and CF5 (Figure 5D) were readily visualized with HRP-EXG:CBM.

EXG:CBM was also visualized with fluorescence using FITC conjugated to avidin. Again, a highly specific staining was detected in paraffin embedded lung tissue from mice exposed by aspiration to CF1 (Figure 6A). The CF1 in Figure 6A was faintly visible under light microscopy (Figure 6B). No signal was detected in lung sections from vehicle exposed mice (Figure 6C). EXG:CBM was also visualized with fluorescence in cryopreserved tissue slides from mice exposed to CF3 (Figure 7A) and CF1 (Figure 7B).

Figure 5. HRP-EXG:CBM staining of paraffin embedded lung sections from mice exposed to 40 μg of cellulose fibers and counterstained with Mayer’s hematoxylin. Microscope objective is 10×. A, CF2; B, CF3; C, CF4; and D, CF5. Arrows locate cellulose fibers identified by the EXG:CBM staining.

■ DISCUSSION

The understanding of how exposure is related to biodistribution and the internal dose is crucial in the evaluation of adverse effects of hazardous agents. In nanotoxicology, detection of nanomaterials in biological tissues is often hampered either by high background levels (e.g., zinc oxide, iron oxide, and others) or by the lack of sensitive and specific detection methods of the nanomaterials. Nanomaterials may be visualized using electron microscopy provided that they are electron dense16,17 or may be recognized by their specific structures.18,19 Other detection methods such as dark field hyperspectral microscopy have been used to visualize nanomaterials.20−22 We have taken an alternative approach and utilized the specific binding properties of a CBM of a cellulose degrading enzyme to develop highly specific visualization of cellulose fibers.

We demonstrate that the detection method can be used to detect and visualize different kinds of cellulose fibers including four different nanofibrillar celluloses. The binding assay is simple and robust and may be used on both cryopreserved and paraffin-embedded tissues. The level of nonspecific staining is very low, the staining procedure is straightforward, and the assay has good sensitivity. The $K_d$ of the EXG:CBM binding to nanofibrillar cellulose is in the order of $10^{-8}$ M, thus in the same range as the antibody−antigen interaction. Furthermore, in contrast to many antibodies, EXG:CBM-binding is not compromised by formaldehyde treatment, and EXG:CBM recognizes both unmodified cellulose and cellulose fibers which have been modified by carboxylation and methylation (such as CF2.
and CF3). Thus, the binding of EXG:CBM is quantitative and robust. The fact that para-embedding does not interfere with the assay furthermore opens a wide variety of possible applications including detection of cellulose fibers in preserved animal tissues, tissues from patients, and occupationally exposed individuals.

On the basis of previous investigations of the interaction of EXG:CBM with cellulosic materials, the application of EXG:CBM can likely be extended to the detection of compounds like bacterial microcrystalline cellulose, Avicel, and α-chitin (a β-1,4 polymer of 2-N-acetyl-glucosamine units).10 The affinity of EXG to Avicel was exploited in the present purification of EXG. The β-1,4-glycanase from Cellulomonas fimi from which EXG:CBM is derived has been shown to hydrolyze para-nitrophenyl-β-D-xylobioside showing that the enzyme has affinity for xylans (hemicellulose) in the active site. However, this observation cannot be extended to describe the binding specificity of EXG:CBM alone.23

Inhalation of cellulose fibers in cotton dust has been associated with respiratory disease.6 Wood dust has been classified as a human carcinogen that can cause adenocarcinomas of nose and paranasal sinuses and occupational exposure to wood dust is associated with highly increased risk of sinonasal cancer.7,24,25 Since there is no relevant model for testing the carcinogenicity of wood dust in animals, the carcinogenic component in wood dust is still unknown. The very long latency between exposure and cancer could indicate that long-term retention of wood-dust components is important and might point to cellulose.24 A method for specific detection of cellulose is critical for understanding the distribution and retention of inhaled cellulose. The binding data for all of the studied cellulose fibers in the present study (Table 2) show quantitative binding of EXG:CBM to all of the studied cellulose fibers. The high specificity for cellulose of our detection method allows for the development of assays for quantitative and qualitative detection of nano-fibrillar cellulose. The EXG:CBM was biotinylated on two sites, and both mono- and dibiotinylated EXG:CBM were detected (Figure 1B). However, a uniform double-labeling of EXG:CBM should be achievable by increasing the excess amount of NHS-biotin used.

In a study of cellulose toxicity, Cullen et al. did not succeed in locating cellulose fibers in macrophages after a 14-day inhalation exposure to cellulose fibers or after a 28 day recovery period, probably because the low refraction index of cellulose prevented its visualization in optical microscopy.24 Using the

Figure 6. Paraffin embedded lung sections of mice exposed to CF1 or vehicle for 24 h. A: Lung section from a mouse exposed to 200 μg of CF1 by aspiration (ASP). The section was stained with biotinylated EXG:CBM and FITC fluorescent avidin. Microscope objective is 40×. Arrows locate cellulose fibers identified by the EXG:CBM staining. B: The same tissue section in bright field with no counterstaining. CF1 is located by arrows. Microscope objective is 40×. C: Lung section from a vehicle-exposed mouse stained with biotinylated EXG:CBM and FITC fluorescent avidin.

Figure 7. Cryopreserved lung sections of mice exposed for 24 h. A: Lung section from a mouse exposed to 200 μg of CF3 by aspiration. The section was stained with biotinylated EXG:CBM and FITC fluorescent avidin. Microscope objective is 40×. Arrows locate cellulose fibers identified by the EXG:CBM staining. B: The same tissue section in bright field with no counterstaining. CF1 is located by arrows. Microscope objective is 40×. C: Lung section from a vehicle-exposed mouse stained with biotinylated EXG:CBM and FITC fluorescent avidin.
methods described herein, cellulose fibers were faintly visible in H&E staining as can be seen by comparing Figure 2A and B or Figure 3C and D. In other work, microcrystalline cellulose has been visualized with a modified Russell Movat penta-chrome stain.26 However, the staining procedure was characterized as “technically challenging” by the authors due to inconsistency of the staining results and due to additional staining of tissue collagen leading to potential misinterpretations.26 We believe that the presently described method is a promising tool for specific visualization of cellulose fibers in future in vivo experiments, due to the high specificity and robustness of the assay.

**CONCLUSIONS**

In conclusion, we have described a novel, robust method for the specific detection of cellulose fibers including four different nanofibrillar cellulosics. The binding to cellulose fibers was quantitative, and the method can be used to visualize cellulose in biological tissue with high sensitivity and specificity. The quantitative binding to cellulose may allow for the development of quantitative detection assays.

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**Notes**

The authors declare the following competing financial interest(s): I.W. is employed by Stora Enso, and M.N. is employed by UPM Kymmene Oyj, producers of nanofibrillar cellulose.

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**ABBREVIATIONS**

ASP, aspiration; B

\textsubscript{max}, concentration of binding sites; CF1–5, cellulose fibrillar 1–5; E. coli, Escherichia coli; EXG:CBM, carbohydrate binding module of β-1,4-glycanase (EXG); HCl, hydrochloric acid; H&E, hematoxylin and eosin; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; K

\textsubscript{D1–5}, indicates the binding constant for 1-site binding or the 2-site binding; [L], concentration of free (not bound) EXG:CBM; NHS-biotin, N-hydroxysuccinimidobiotin; MALDI-TOF MS, matrix-assisted laser desorption/ionization—time of flight mass spectrometry; O.C.T. compound, optimal cutting temperature compound; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TFA, trifluoroacetic acid.

**REFERENCES**


**NOTE ADDED AFTER ASAP PUBLICATION**

This paper was published ASAP on August 6, 2015, with an error in the spelling of the name of author Harri Alenius. The corrected version was reposted on August 17, 2015.