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The need to freeze - dehydration during specimen preparation for electron microscopy collapses the endothelial glycocalyx regardless of fixation method

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Abstract

Objective: The endothelial glycocalyx covers the luminal surface of the endothelium and plays key roles in vascular function. Despite its biological importance the ideal visualisation techniques are lacking. The current study aimed to improve the preservation and subsequent imaging quality of the endothelial glycocalyx.

Methods: In mice, the endothelial glycocalyx was contrasted with a mixture of Lanthanum and Dysprosium (LaDy). Standard chemical fixation was compared with high pressure frozen specimens processed with freeze substitution. Also, isolated brain microvessels and cultured endothelial cells were high pressure frozen and by transmission soft x-ray imaged under cryogenic conditions.

Results: The endothelial glycocalyx was in some tissues significantly more voluminous from chemically fixed specimens compared with high-pressure frozen specimens. LaDy labelling introduced excessive absorption contrast which impeded glycocalyx measurements in isolated brain microvessels when using transmission soft x-rays. In non-contrasted vessels, the glycocalyx was not resolved. LaDy-contrasted, cultured brain endothelial cells allowed to assess glycocalyx volume *in vitro*.

Conclusions: Both chemical and cryogenic fixation followed by dehydration lead to substantial collapse of the glycocalyx. Cryogenic fixation without freeze substitution could be a way forward although transmission soft x-ray tomography based solely on amplitude contrast seems unsuitable.

Keywords: Endothelial glycocalyx, high pressure freezing, soft x-ray imaging, electron microscopy

Introduction

Cells are covered by a complex layer of extracellular carbohydrates termed the glycocalyx. The luminal surface of endothelial cells are lined by a thick coat of glycocalyx, which is important for maintaining endothelial function. It shields adhesion proteins and accordingly inhibits undesirable binding of leukocytes and platelets under homeostatic conditions. Furthermore, the endothelial glycocalyx decreases trans-endothelial permeability, acts as a mechanosensor and as a matrix for heparan sulfate-binding proteins¹⁻⁴.

Its importance in vascular pathophysiology is increasingly being appreciated and judging from experimental models it is involved in several lifestyle-related diseases^{5,6} as well as in infectious diseases⁷⁻⁹. Importantly, several of these findings are also supported by clinical observations¹⁰⁻¹⁴.

Previous studies have documented that there is considerable difference in glycocalyx size when one compares data obtained by *in vivo* imaging with data obtained *ex vivo* by the use of electron microscopy^{1,15}. One potential explanation for this difference is the chemical fixation and dehydration employed during standard electron microscopy preparation procedures since these procedures have been suggested to lead to a pronounced collapse of the glycocalyx and accordingly may lead to gross underestimation of its volume and appearance^{1,16,17}. Also, doubts have been raised about the use of *in vitro* cell culture in glycocalyx studies of endothelial cells since the glycocalyx is reduced or has lost its physiological properties^{18,19}. *In vitro*, its presence seem to depend on the addition of albumin and serum to the cultivation medium and also seems to depend on fibronectin as a matrix^{16,20,21}. By using atomic force microscopy, *in vitro* growth of glycocalyx has been shown to slowly develop over time. Consequently, a too short cultivation time may lead to a premature glycocalyx in experimental setups^{22,23}.

Accordingly, a comparison of different endothelial glycocalyx fixation methods has been warranted. The present study uses high pressure freezing (HPF) followed by freeze substitution and transmission electron microscopy (TEM) imaging to compare the endothelial glycocalyx *ex vivo* in mice and compare with traditional chemical fixation. Furthermore, a novel method using soft x-ray tomography (SXT) on cryo-preserved *in vitro*-grown endothelial cells and isolated brain capillaries is described. In cryo SXT, biological specimens are kept in their hydrated state and samples can be imaged without adding contrast agents thereby providing a method to study submicron structures in cells and tissues²⁴⁻²⁶. By comparing different methods, we can gain insight on how the endothelial glycocalyx appears under different imaging conditions.

Materials and methods

Chemicals

0.5% (w/v) Lanthanum nitrate (Electron Microscopy Sciences (EMS), Hatfield, PA, USA) and 0.5% (w/v) dysprosium acetate (EMS) was mixed (LaDy²⁷) and dissolved in 0.9% saline (substrate department, University of Copenhagen).

Murine studies

All animal studies and protocols were approved by the Danish animal inspectorate (Danish Ministry of Justice) as well as by the veterinarians at University of Copenhagen, where animals were housed. All methods were carried out following Danish guidelines for animal studies. Female Balb/c mice (Taconic, Denmark) aged 5-7 weeks were enrolled in the study. Animals had unlimited access to food and water and kept at a 12:12 h day and night cycle. In deep anaesthesia (Hypnorm/Midazolam (Fentanyl 788 µg/kg, fluanisole 25 mg/kg, 12.5 mg/kg), s.c.), the thoracic cavity was opened, and the mice were transcardially perfused; the perfusate depended on the experiment (figure 1).

For electron microscopy mice were either perfused with 20 ml LaDy dissolved in 0.9% saline (cryogenic preservation) or with 10 ml LaDy dissolved in 0.9% saline followed by 20 ml LaDy dissolved in 2.5% glutaraldehyde in 0.05 M HEPES buffer (EMS). Control mice were chemically or cryogenically fixed as described above but mice were not perfused with LaDy. For soft x-ray tomography, mice were in deep anaesthesia transcardially perfused with 20 ml isotonic saline with or without LaDy and brain capillaries were isolated by dextran-based capillary depletion or by the use of cell strainers (40 and 100 µm, Corning, Sigma-Aldrich) as previously described²⁸. Some mice had fluorescein-conjugated wheat agglutinin lectin injected i.v. prior to transcardial perfusion (100 µg lectin/mouse in 100 µl PBS). An overview is found in figure 1. In total, 16 mice were used for EM studies (4 mice pr. group: HPF vs. chemical fixation, LaDy vs. no contrast) and 6 mice were used for soft x-ray tomography.

Capillary depletion

Capillary depletion was performed as described previously²⁹. In short, after perfusion the brain was isolated and one hemisphere was homogenised four times using the loose pestle in 1 ml cold HEPES buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, and 10 mM glucose at pH 7.4) using a Dounce grinder (EMS). Next, 1 ml of cold dextran solution (37% w/v, 4 kda, Sigma-Aldrich, Denmark) was added followed by four additional strokes with the loose pestle. The homogenate was transferred to Eppendorf tubes and centrifuged for 20 minutes at 5400x g at 4°C. The lipid cake and the supernatant were carefully removed and 1 µl of the pellet was then applied to an EM grid (hexagonal, Gilder grids, Grantham, UK) and high pressure frozen (HPM100, without ethanol as synchronisation fluid, Leica).

Cell culture

Primary human brain microvascular endothelial cells (ScienCell Research laboratories, CA, USA) were cultured in endothelial cell medium (ScienCell Research Laboratories) supplemented with 5 % FBS, endothelial cell growth supplement (ScienCell Research Laboratories), Penicillin (100 U/ml) and streptomycin (100 µg/ml) (ScienCell

Research Laboratories). Passage 4-6 was used. Also, murine, immortalized brain endothelial cells were employed (bEND.3, ATCC). These were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and antibiotics as described above. Passage 18-25 was used.

Cells were grown directly on Fibronectin-coated gold grids (Quantifoil R2/2, mesh 200, Jena, Germany). The cells were seeded in low density (2×10^5 cells/ml) to ensure sufficient time to develop a representative glycocalyx²². Just before fixation cells were stained with LaDy or left unstained. Staining was performed for 5 min before excess was removed by flushing with solvent. Some cells were also stained with fluorescein-conjugated wheat germ agglutinin (10 µg/ml for 10 minutes) prior to cryo-fixation.

Cryogenic fixation

All cryogenic fixation was performed by high pressure freezing (HPF). HPF enables full vitrification within milliseconds of biological specimens that have a higher thickness as compared to what is seen for plunge freezing³⁰. Cooling rates for the reported specimens exceeded 15000 K/s. Tissue for TEM was quickly removed from the transcardially perfused mice, sliced to a thickness of maximum 200 µm and cut to fit 3 mm diameter planchets prior to HPF. 1-Hexadecene (Sigma-Aldrich) was used as cryo protectant for TEM.

TEM grids containing cells and isolated capillaries for SXT were placed on 6 mm planchets and cryo-preserved without any additional fluid and the cavity facing outwards in order to keep the height of the sample as small as possible.

Freeze substitution

Tissue was stored in liquid nitrogen until freeze substitution was initiated. Freeze substitution was performed with Leica AFS2 (Leica). Freeze substitution was initialised in 1% OsO₄ in acetone at -90°C for 48 hours followed by a gradual increase in temperature up to -30°C for 9 hours. Specimens were subsequently rinsed in acetone for 1 h three times. The temperature was slowly increased to room temperature and finally the specimens were embedded in Epon (Taab 812, Berkshire, UK) according to standard procedures⁷.

Chemical fixation

Mice were perfused with either LaDy or the respective solvents without LaDy. Following an initial stain, the mice were furthermore perfused with 2.5% glutaraldehyde in 0.05 M HEPES buffer (pH=7.4) plus either LaDy (0.5% and 0.5%) or no additive for 5 minutes. Next, several tissues were removed and immersed in the respective primary fixative for 2 hours before transfer to 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH=7.4).

Tissue was punched with a biopsy needle (diameter = 1 mm, Integra Miltex, Saint Priest, France) prior to osmification and dehydration. Tissue was osmicated for 60 minutes (1% in 0.1 M cacodylate buffer, pH=7.4, EMS) and subsequently dehydrated according to standard procedures. Tissue was embedded in Epon resin (TAAB 812, Berkshire, UK). Ultrathin 60 nm thin sections were post stained with lead citrate and uranyl acetate as previously described ³.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed using CM100 (Philips, Eindhoven, Holland) operated at 80 kV. At least 10 vessels per mouse was analysed. Glycocalyx thickness was measured using FiJi ³¹. Images were recorded with an Olympus Veleta camera (Olympus, Tokyo, Japan) and the iTEM software package.

Soft x-ray tomography

Grids were stored in liquid nitrogen until being analysed. Soft x-ray tomography (SXT) imaging of brain endothelial cells and isolated microvessels was carried out at the energies 510 or 520 eV within the water window (284-537 eV i.e. between the x-ray absorption K-edges of carbon and oxygen) under cryogenic conditions exclusively ²⁶. SXT was performed at the synchrotron light sources at Alba (beamline Mistral, Barcelona, Spain)²⁵ and at BESSY II (beamline U41-TXM, Helmholtz-Zentrum Berlin für Materialien und Energie Elektronenspeicherring, Berlin, Germany) ^{32,33}. Similarities and differences between the two cryo SXT beamlines are highlighted in table 1 ²⁴.

Tomograms for *in vitro* grown cells were collected in steps of 1° over the sample tilt range between -65° - + 65°, exposure time/projection varied between 2-8 seconds/projection. The x-ray dose received was around 10⁸ Gy and thus well below the damage limit at 10⁹ Gy ³⁴. Tomograms for microvessels isolated by capillary depletion were collected in steps of 3° between -51° - +51°, exposure time/projection was 1 second/projection. These settings were empirically determined in order to reduce radiation damage. Tomograms for microvessels isolated by cell strainers were collected in steps of 1°; the exact tilt angles varied from tomogram to tomogram but covered at least 100° rotation in total. Two zone plate lenses were used: a 25 nm at BESSY II and a 40 nm at ALBA. Pixel size: 10-13 nm (table 1).

Tomogram reconstruction and image analysis

Tomograms were aligned using either IMOD ³⁵ or Bsoft ³⁶. Tomogram 3D reconstruction was performed using Tomo3D ³⁷. The following filters were applied Gaussian and Median (sigma=26 nm). Image measurements and annotations were performed using FiJi ³¹.

Statistical analyses

All statistical analyses were performed using R (version 2.15.2 for windows ³⁸). A Mann-Whitney U test was applied. A P-value below 0.05 was considered statistically significant.

Results

The resolved endothelial glycocalyx appear different when comparing tissue after chemically fixation versus after cryogenic fixation

Chemical fixation has been suggested to cause shrinkage of the glycocalyx volume partly due to dehydration ¹⁶. To assess this change, in brain tissue, chemical fixation was compared with HPF followed by freeze substitution of tissues stained with lanthanides. Chemical fixation and staining with a mixture of Lanthanum and Dysprosium (LaDy) resulted in a voluminous glycocalyx spanning more than 100 nm from the luminal surface (110 ± 26 nm (standard deviation (SD))), table 2, Fig. 2 A, B). As previously described in detail ²⁷, LaDy staining resulted in a dense glycocalyx enabling highly detailed visualisation of the glycocalyx structure. HPF-preserved tissue contrasted with LaDy also resulted in a glycocalyx resolving a high level of details as seen in chemically fixed tissues. Chemical fixation rendered a significantly more voluminous glycocalyx as when compared to HPF brain tissue (39 ± 8 nm, $p < 0.001$, table 2, Fig. 2 C, D). Tissue not contrasted with LaDy did not resolve a glycocalyx (Fig. 2E).

In heart and lung tissue, LaDy staining also resulted in a dense and voluminous staining regardless of fixation method (Fig. 3, table 2). When comparing endothelial glycocalyx dimensions in lung tissue, the glycocalyx seemed less voluminous in HPF processed tissue as compared with chemically fixed tissue but this difference was not significant ($p = 0.1$, table 2).

Cryogenic SXT can visualise the glycocalyx after LaDy contrasting

Since both fixation methods involve an exchange of water with organic solvents, we tested a new method to assess the endothelial glycocalyx. Cryo SXT allows imaging of entire cells in their native, hydrated state without introduction of any dyes or chemicals. This is possible due to selection of the x-ray energy within the so-called “water window” range in which oxygen atoms are nearly transparent to x-rays while carbon atoms are strongly absorbing. In contrast to electrons, which can penetrate up to approximately 1 μ m deep into biological specimens ¹⁷, soft x-rays penetrate well up to around 10 μ m in depth ³⁹. The cerebral microvessels are of approximately such diameter and were isolated using the capillary depletion technique with the outcome of small segments of cerebral microvessels (Fig. 4A). Areas were selected in which series of x-ray transmission images were acquired over a range of tilts (Fig. 4B shows one example of a zero-degree tilt transmission image). Three-dimensional maps of the scanned areas were reconstructed from these tilt-series of transmission images. In tomograms of selected areas, tight junctions and intracellular organelles are visible (Fig. 4C). However, the presence of dextran particles in these preparations absorbed substantial amounts of x-

rays and in order to reduce beam damage to a minimum we reduced exposure time/angle and total number of angles. Accordingly, 3D reconstructions were of low contrast (supplementary video 1). Various filters were applied to aid in resolving intracellular details and a Gaussian filter (sigma=26 nm) proved to result in the highest level of contrast yet causing difficulties in resolving intracellular structures to any acceptable high level of details. Without the addition of any contrasting agent, the glycocalyx was not resolved. In some vessel segments erythrocytes were trapped, enabling an estimation of the cell free layer in orthogonal views (Fig 4D, supplementary movie 2). The cell free layer was measured to be 438 nm thick (median, range 176-580 nm). In preparations without dextran-based isolation, only minor beam damage was observed.

The presence of lanthanides on the luminal surfaces after LaDy perfusion resulted in poor penetration (high absorption) of x-rays and accordingly very dark vessel segments (Fig. 5). In these segments, no information on either glycocalyx dimensions or cell free layer could be obtained. Thus, in these preparations of isolated, cerebral microvessels, the glycocalyx was not resolvable using SXT.

Instead we turned to imaging cryopreserved *in vitro* grown endothelial cells, which enabled the visualisation of intercellular organelles at high detail (Fig. 6A). Monolayers of endothelial cells are below one micron in thickness and are thus ideally suited for SXT allowing for well-exposed tilt series without beam damage. Intracellular lipid bodies were particularly conspicuous while mitochondria were annotated by their cristae (Fig. 6A, B). However, as seen in isolated brain capillaries, luminal carbohydrates did not result in sufficient contrast in the “water window” and instead a bare plasma membrane was evident (Fig. 6A, B). Contrasting with LaDy ²⁷ resulted in a dense glycocalyx covering parts of the plasma membrane, while some areas appeared like non-contrasted plasma membrane (Fig. 6C-D). Staining with LaDy turns the smooth plasma membrane into a thick, rough surface indicating binding to negatively charged glycans. As described above, LaDy contrasting also resulted in considerable absorption of photons impeding reconstructions of tomograms. In the areas present, the glycocalyx had a height of approximately 98 ± 12 nm. Wheat germ agglutinin was also investigated as a possible contrast agent but despite its strong affinity to the glycocalyx it did not resolve the glycocalyx.

Discussion

Previous studies have extensively discussed the volume of the endothelial glycocalyx and the effects of chemical fixation^{1,16}. In terms of glycocalyx volume, a discrepancy exists between methods quantifying glycocalyx dimensions *ex vivo* using TEM versus methods using microparticle-based measures *in vivo* and *in vivo* lectin-labelling^{1,5,40}. This could be the result of shrinkage due to glutaraldehyde fixation or dehydration⁴¹ or a mere collapse of the hydrated structure. Recently, a review mentioned that HPF tissue can be successfully post-stained for specific labelling of the glycocalyx during freeze substitution⁴² but this method has not been assessed in detail. In our study it could be shown that HPF indeed is applicable for this type of study. To address the effect of chemical fixation, the volume of LaDy-contrasted glycocalyx in brain capillaries processed by conventional chemical fixation was compared with HPF-processed tissue. However, when we compared chemically fixed tissue with HPF and freeze substituted tissue the chemically-fixed tissue did not appear collapsed to a greater extent. In contrast a somewhat more voluminous glycocalyx was noticed in chemically fixed tissue. This was particularly prominent in brain vessels. This could be a result from the HPF itself but since it was not seen for vessels in lung tissue this seems unlikely.

The appearance of extracellular polymers is sensitive to changes in the environment¹⁷. One explanation for the small glycocalyx seen in HPF and freeze substituted tissue is that similarly to specimen preparation for chemically-fixed tissue, water is removed and exchanged with organic solvents. The Hildebrand solubility parameter suggest that organic solvents including ethanol and acetone are poor solvents for glycosaminoglycans^{43,44} and accordingly a collapse is inevitable.. In fact, acetone may be an even more poor solvent than ethanol for glycosaminoglycans leading to an even greater collapse. The Flory theory describes that the solvent affects polymer swelling (volume) and thus affects the extent it stretches when tethered to a surface like a plasma membrane on cells⁴⁵. This behaviour depends on how energetically favourable the interaction between the polymer chains is compared with its interactions with the solvent. For example, if the polymers are put into a poor solvent they prefer interacting with other polymers and thus collapse. The level of collapse depends on the number of monomers in a given polymer. Each disaccharide repeat is considered as a monomer unit and is approximately the length of 1 nm for GAGs⁴⁶. The length (r) scales with the number of disaccharide repeats (N) as a function of the Flory exponent x ($r \sim N^x$), which is approximately 0.6 for a good solvent and 0.33 for a poor solvent⁴⁷. One example is HA that can have up to 25000 repeats⁴⁶, which means that a polymer can be stretched up to approximately 435 nm in a good solvent and collapse and have an end-to-end distance of around 28 nm in a poor solvent. Yet, it can not be ruled out that the binding of LaDy to negatively charged glycosaminoglycans (GAGs) prior to initiation of fixation and dehydration to some extent can stabilise the labile GAG structures.

By performing SXT microscopy, one can image cells without exchanging solvents²⁶ and since resolution lies in the interface between light and electron microscopy it offers the possibility to obtain 3D information of cellular and

subcellular structures⁴⁸⁻⁵⁰. This contrast is obtained without the use of either fixation or contrast agents. In the “water window” energy range there is a large difference in x-ray absorption when comparing water (containing oxygen) with organic compounds (carbon-rich) such as lipids and proteins; this difference results in contrasted images^{26,49}. SXT of a contrasted glycocalyx has not previously been performed. We used LaDy and its interaction with anionic GAGs to visualise the endothelial glycocalyx as undisturbed and native as possible. In segments of isolated brain capillaries, the absorption was too high to resolve any cellular structures. Thus, the x-rays could not penetrate LaDy-labelled microvessels and resolve the endothelial glycocalyx. Since 10 μm is the maximum depth of sufficient penetration of SXT as well as an approximate diameter for cerebral microvessels adding LaDy and this additional contrast hampers imaging of the *ex vivo* glycocalyx. The glycocalyx was not sufficiently dense to be seen in unlabelled vessels since feature size resolved by SXT (~ 30 nm half pitch^{26,51}) is somewhat larger than the thickness of single GAG chains (0.5-1 nm)^{26,49,52-54}. In areas of trapped erythrocytes, the cell free layer was measured at high resolution supporting the previous findings of a cell free layer of several hundred nanometers even in small capillaries^{1,55}. An alternative to using amplitude contrast for SXT imaging of the glycocalyx could be to use phase contrast SXT as this method previously has shown to result in higher contrast⁵⁶.

In endothelial monolayers LaDy worked well as a contrast agent as has been seen for TEM. We found uneven labelling of the *in vitro* grown cells giving a possible explanation for the lack of functional glycocalyx in conventional tissue culture systems^{18,21,57}. Also, when using SXT the glycocalyx was present in tufts yet extending comparable distance from the bilayer membrane as noticed *in vivo* and in correspondence with previous *in vitro* studies²³.

One unexpected finding was the large absorption of x-rays by dextran particles. This led to the need to make large tilt intervals to avoid beam damage resulting in less well-defined reconstructions. The reason for the high beam damage likely results from the chemical structure of carbohydrates making them less able to pack closely compared with e.g. lipids in plasma membranes.

In this study, cryo SXT was performed at two different synchrotrons and the resulting data was merged. It can be argued that using two different synchrotrons can introduce some level of variation. Yet, we expect this variation to be minimal since the imaging setup at the two beamlines is extremely similar (table 1)²⁴. Initially the beamline at Bessy II used specially designed grids, but now normal EM grids can be used as well²⁶.

The results obtained also suggest that the previously reported 11 μm thick glycocalyx¹⁶ may arise from something quite unrelated to the glycocalyx *per se*. In the paper by Ebong *et al.*¹⁶ no contrasting agent was used and in none of the cryo-preserved specimens for neither TEM nor SXT no such structure is present. Accordingly, the reported matrix is most likely an artefact resulting from the methodology (slam freezing) and the glycocalyx dimensions thus does not seem to exceed what has been measured with *in vivo* imaging^{1,58}.

In the brain we found the glycocalyx to be less voluminous than what has previously been reported in the kidney, brain and umbilical cord when using TEM ^{27,42,57,59}. We expect this to be a matter of biological variation as well as choice of staining protocol since an alkaline buffer was not used in the present study ⁵⁹.

In vivo imaging has documented that a layer of several hundred nanometers to a few microns exist in which larger molecules and micron-sized particles can not penetrate ^{1,18,55,60,61}. This view is supported by assessing albumin interactions with the glycocalyx by using fluorescence correlation spectroscopy and electron spin resonance ^{62,63}. Modelling studies has quantitated how sparse a matrix is needed in order to bind plasma proteins that can maintain an elevated colloidal osmotic pressure and this way form an incompressible layer ⁶⁴. Seemingly, this sparse matrix is difficult to resolve *ex vivo* using high resolution imaging.

In conclusion, the results show that lanthanides give substantial contrast to the endothelial glycocalyx regardless of fixation method and also works as a contrast agent in SXT microscopy. Yet it is concluded that chemical fixation does not lead to a more pronounced collapse of the endothelial glycocalyx as when compared with HPF tissue. Instead, the data support that post fixation the glycocalyx during dehydration collapses into a dense surface layer regardless of the fixation method. As was shown for bacteria, imaging the glycocalyx in a frozen, hydrated state would be ideal ¹⁷. Cryogenic SXT allows for imaging in hydrated state and although the LaDy stained *ex vivo* preparations seem too dense for glycocalyx imaging using the studied sample preparation techniques, SXT may work well for other sample preparations.

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Author contributions

CH performed *in vitro* and *in vivo* experiments, performed TEM, performed HPF, performed SXT, designed the study and wrote the manuscript.

SK performed SXT, tomography, interpreted results and assisted in writing the manuscript.

SW, PG, AJPB and EP performed SXT, alignment of tomograms and supported the writing of the manuscript.

KQ performed HPF, interpreted the TEM results and assisted in writing the manuscript.

TLA participated in study design and in writing of the manuscript.

All authors reviewed and accepted the final version of the manuscript.

Additional information

Competing interests

The authors declare no competing financial or non-financial interests.

Table 1. Comparison of beamlines used for SXT.

Beamline	X-ray source	Zone plate	Monochromator	Photon energy
U41-TXM (Berlin)	Undulator	25 nm	Blazed plane grating	510 eV
MISTRAL (Barcelona)	Bending magnet	40 nm	Variable -spaced line grating	520 eV

Table 2. Comparison of glycocalyx dimensions in different tissues by using different fixation methods. Dimensions quantified by using TEM. SD: Standard deviation; ND: Not determined.

Tissue	Fixation	Average size ± SD [nm] per group	Average size ± SD [nm] per animal	Average size ± SD [nm] per vessel
Brain	Chemical	110±26	108±28	111±22
	HPF	39±8	41±10	42±10
Heart	Chemical	121 ±35	122±42	120±34
	HPF	ND	ND	ND
Lung	Chemical	64±15	67±15	66±13
	HPF	40±11	40±13	38±11

Figures and figure legends

Figure 1

Overview of experimental procedures and approaches to visualise the endothelial glycocalyx. The endothelial glycocalyx in BALB/c mice was visualised with TEM after either chemical fixation or after high pressure freezing and freeze substitution. An alternative approach using soft x-ray tomography on vitrified specimens was also used.

Figure 2

Endothelial glycocalyx in brain vessels is visible following chemical fixation and high pressure freezing. The glycocalyx is visible as a black, dense staining. A) LaDy contrasting of chemically fixed tissue shows a dense layer on the luminal surface of the cerebral vasculature. B) High magnification image of the glycocalyx in brain vessels (boxed area in A). C) High pressure frozen tissue also enabled contrasting of the glycocalyx. D) At high magnification the glycocalyx is seen (boxed area in C). E) Mice that were not perfused with LaDy did not result in contrasted glycocalyx after cryogenic conservation. Scale bars: A, E: 5 μm ; C: 2 μm , B, D: 100 nm.

Figure 3

LaDy perfusion results in contrasting of glycocalyx in other organs including heart and lung tissue. A) Lung tissue, chemical fixation. B) Heart tissue, chemical fixation. C) Lung tissue, high pressure freezing. Scale bars: A, B, C: 200 nm and inserts: 5 μm .

Figure 4

Isolated, cerebral microvessels show only little *intracellular* contrast. A) Overview transmission x-ray image of isolated microvessels. One vessel on the lower right corner contains three erythrocytes (boxed area). B) Detailed image of the three erythrocytes trapped in a capillary. C) Close up of endothelial cells showing tight junctions (arrow head) and an endosome (arrow). The glycocalyx is not visible. D) Orthogonal view of the tomogram reconstruction of an erythrocyte trapped in a capillary. Data collected at the Mistral beamline, Alba light source. Scale bars: A: 10 μm , B: 2 μm , C and D: 1 μm .

Figure 5

Low-dose x-ray SXT slice of a reconstructed segment of a capillary after LaDy contrasting. A 3D reconstructed vessel segment shows substantial contrast on the vessel wall (arrow) and a very dark lumen (arrow head) due to poor penetration (high absorption) of photons. Data collected at the U41-TXM beamline, BESSY II light source. Scale bar: 2 μm .

Figure 6

Reconstructed slices from cryo SXT of cultured endothelial cells. A) The nucleus (N) is visible as well as multiple endosomes, mitochondria and lysosomes. Lipid bodies (L) absorb x-rays notably. The plasma membrane is marked by an arrow. B) LaDy contrasting resulted in a denser membrane labelling (arrow head), while in some areas of the plasma membrane it appeared like a non-labelled plasma membrane (arrow) (2D projection). C) Focused 2D projection from tilt series of plasma membrane with notable glycocalyx labelling (arrow heads). Data collected at the Mistral beamline, Alba light source. Scale bars: A, B: 1 μm and C: 500 nm.

Supplementary movie 1

Coarsely aligned tilt series of an isolated microvessel imaged with SXT. The microvessel is seen without any cellular details revealed. The dextran beads are associated with the microvessel and absorb high amounts of x-rays leading to beam damage (seen as white, contrasting stripes) in the end of the tilt series. This tilt series consists of 131 projections (-65° - $+65^\circ$).

Supplementary movie 2

Coarsely aligned tilt series of an isolated microvessel imaged with SXT. Three trapped erythrocytes are seen inside the vessel lumen. No beam damage is seen in this capture. The tilt series consists of 96 projections (-50° - $+45^\circ$).

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