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Short communication

APTS-labeled dextran ladder: A novel tool to characterize cell layer tightness

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Abstract

The aim of this work was the development of an easy manageable analytic system for describing tightness of cell layers in a molecular size dependent manner, which is more precise than currently used ones. Dextrans were labeled by reductive amination with fluorescent 1-aminopyrene-3,6,8-trisulfonate (APTS). This mixture, including internal standard diazepam, was used for transport studies, which were accomplished with an established transwell blood–brain barrier model culturing an immortalized porcine brain microvascular endothelial cell line (PBMEC/C1-2). Samples were analyzed by fluorescence measurements, capillary electrophoresis and RP-LC. Following this approach, a permeability pattern could be achieved including each single fraction from APTS, APTS-glucose to APTS-dextran consisting of 31 glucose units. Permeability coefficients were calculated and ranged from 16.38 ± 3.79 μm/min for APTS to 6.07 ± 1.23 μm/min for the APTS-dextran with 31 glucose units (diazepam: 67.97 ± 7.32 μm/min). All in all, the developed APTS-dextran ladder is an useful tool to characterize cell layer tightness – especially to describe paracellular transport ways and leakiness status of the blood–brain barrier over time – applying a wide range from smaller to larger molecules at the same time in order to refine, e.g. TEER, sucrose or Evans blue measurements.
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1. Introduction

Development of new drugs acting on the central nervous system (CNS) is one of the major tasks in pharmaceutical sciences. In the last decades research mainly focused on search for novel drug targets and design of novel drugs rather than on drug delivery [1]. Nevertheless, CNS targeting drugs have to cross the blood–brain barrier (BBB). The BBB maintains the homeostasis of the brain microenvironment, which is crucial for neuronal activity and function. Brain microvascular endothelial cells (BMEC) that constitute the BBB are responsible for the transport of nourishment to neurons and clearance of potentially toxic substances from the brain. Unlike peripheral endothelium, BMEC are characterized by the presence of tight intercellular junctions, minimal pinocytotic activity and the absence of fenestrations [2]. Bands of tight junctions (zonula occludens), between adjacent endothelial cells restrict the paracellular pathway and effectively prevent the passage of polar, hydrophilic drugs between endothelial cells, whereas in non-barrier forming capillaries the bands contain focal discontinuities. This greater tightness of cerebral capillary junctions is reflected in high transendothelial resistances. Typically, electrical resistance values of about 2000 $\Omega\,cm^2$ [3] are observed in pial microvessels on the surface of the brain, compared to 1–3 $\Omega\,cm^2$ in mesenteric capillaries [4]. In order to describe the tightness status of the BBB in vivo or in vitro, next to transendothelial electrical resistance (TEER), determining the permeability of sucrose, Evans blue and FITC labeled dextrans (FD) became the methods of choice. At least since the late 1980s it has been known that commercially available FDs contain various amounts of free FITC [5] and consist of a wide range of polymers with different molecular weights. Commonly, total fluorescence values have been measured in permeation studies without considering the size of the different fluorescent fractions. If leakiness of the BBB occurs – for instance due to lack of fully functional tight junctions – TEER shows a decrease, whereas permeability of sucrose, Evans blue and FDs should increase. In this context, relationship between molecular size and permeability through

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a cell layer at a distinct tightness status are not well described. Several attempts were made to use different types of molecules with different molecular weight for simultaneous studies, but no method for measuring a permeability pattern based on molecular size of similar molecules has been developed yet. Obviously, dextran, a glucose polymer, seems to be an adequate target for designing such a kind of molecular size ladder. Due to their multifunctionality, labeling can be easily achieved either at the hydroxyl or at the carbonyl group. Furthermore, they are cheap and easy to obtain.

2. Materials and methods

2.1. Fluorescence measurements

Total fluorescence values of APTS-labeled dextran were determined with plate reader polarstar galaxy (BMG Labtech, Germany) at an excitation wavelength of 485 nm and an emission filter at 520 nm.

2.2. Equipment for capillary electrophoresis (CE)

P/ACE 5500 system with a LIF detector (Beckman Instruments), Ar laser (excitation at 488 nm, emission wavelength of 520 nm), “System Gold 8.0” software.

At the beginning of each test series the sample solution was measured in triplicates. The mean value of this measurement was used as 100% value (C_A in Section 2.5) for permeability calculations. Typical relative standard deviation (R.S.D.) of peakarea and migration time were for APTS-dextran with 5 glucose units 1.28 and 1.34%, for APTS-dextran with 10 glucose units 2.60 and 1.78%, for APTS-dextran with 15 glucose units 5.77 and 2.20% and for APTS-dextran with 20 glucose units 6.91 and 2.47%. To prove the linearity of the method a dilution series has been performed, showing a linear response between 4.8 and 240 μM for APTS-dextran with 5 glucose units ($R = 0.988$, $y = 52.573x - 401.77$), APTS-dextran with 10 glucose units ($R = 0.992$, $y = 52.2x - 316.92$), APTS-dextran with 15 glucose units ($R = 0.992$, $y = 41.022x - 257.13$) and APTS-dextran with 20 glucose units ($R = 0.989$, $y = 25.652x - 191.29$). The linearity of the applied method has been shown for several monosaccharides before [6]. Further studies on the validation of the method will be part of the validation activities for our complete testing protocol for cell layer tightness.

2.3. Electrophoretic conditions

All separations were performed at 25 °C. The detector was situated 7 cm from the anodic end (reverse polarity), injection by pressure was accomplished with 3.45 kPa for 4.0 s.

FITC-dextran: deactivated fused silica capillary columns (Ziemer Chromatographie, methyl/silyl derivatized, 100 μm I.D., 360 μm O.D., 37 cm length) TBE buffer solution: 44.5 mM Tris, 44.5 mM boric acid, 2 mM EDTA and 0.5% HPMC (Hydroxy-propyl-methyl-cellulose), 20 kV.

APTS-dextran: neutral capillary columns (Beckman Coulter, 50 μm I.D., 47 cm length), buffer solution: 25 mM LiAc (pH 4.75) and 0.4% PEO (molecular weight = 8,000,000), 23.5 kV.

Before every run, the capillary was flushed 1 min with water and then 4 min with the applied separation buffer.

2.4. Labeling reaction

In order to develop a system which enables differentiation between several molecular weight fractions, dextrans (average molecular weight = 6000 g/mol, Fluka) were labeled with APTS by reductive amination and were analyzed by CE. A labeling reaction (Fig. 1) was reported [7–9], which was slightly modified. Dextran solution was not dried, it was dissolved in a total volume of 20 μl. 1 M $NaBH_3CN$ was dissolved in MeOH instead of THF and 5–10 μl were applied depending on the age of the solution. The labeling mixture consisting of 20 μl dextran, 5 μl 0.1 M APTS (in 1 M citric acid) and 5–10 μl 1 M $NaBH_3CN$ was incubated at 85 °C for 3.5 h. The resulting fluorescence values of 240 μM APTS-dextran solutions determined with the plate reader were in average 235,220.33 ± 23,302.31 ($n = 10$). Stability experiments with sterile filtered 240 μM APTS-dextran solutions showed a maximum decrease of fluorescent values of 1.75% when stored at 4 °C for 8 months and no significant decrease when incubated at 37 °C for 2 days. Also, fraction distributions of APTS-dextran solutions of the stability tests were controlled by CE and showed no significant changes within these time ranges. After derivatisation, fraction pattern was checked prior to first application for transport studies.

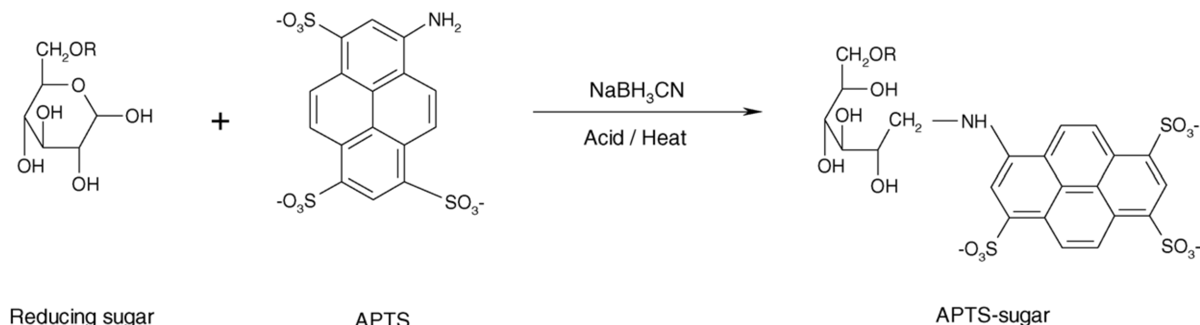

Fig. 1. Scheme of labeling reaction, reductive amination of reducing sugars with APTS.

2.5. Transwell model – transport studies

For permeation experiments porcine brain microvascular endothelial cells (PBMEC/C1-2) [10] were grown to confluence on membrane filter inserts, which were coated with bovine collagen and fibronectin. Medium compositions and culturing conditions were described earlier [11]. After visual control of cell monolayers using light microscopy and determining TEER, transport studies with APTS-dextran 6000 (240 μM in culturing medium) were accomplished like described elsewhere [12]. TEER was measured with a Millipore Millicell Electrical Resistance System (ERS, Millipore Vienna). Electrodes were placed into the insert and the well and the meter was used to record TEER, which was derived from Ohm's law where $V=IR$ (V, voltage; I, current; R, resistance). Resistance is inversely proportional to permeability, and in this instance reflects permeability to small ions that carry electrical charge. Control measurements from blank inserts were subtracted to calculate TEER for PBMEC/C1-2 cell layer. Resistance values were multiplied by the surface area of the insert (4.2 cm^2) to express them in $\Omega\,cm^2$. TEER was monitored daily and was determined after medium exchange. APTS-labeled dextran was used to characterize the paracellular transport route. In order to describe transcellular transport as well, the lipophilic substance diazepam was chosen as an internal standard. Diazepam is supposed to permeate across the BBB by passive diffusion [13,14] and not by an active transport system. Thus, diazepam is a suitable standard substance to compare transcellular permeability across several in vitro models in general and in our case to the paracellular permeability of APTS-dextran. Diazepam was added at concentrations of 50 μM to APTS-dextran test solutions.

Inserts were transferred at several time intervals in further wells, which were filled with prewarmed medium, in order to maintain sink conditions. At the end of transport studies, supernatant of the inserts was removed, inserts were transferred into wells with fresh medium and the apical chamber was refilled with medium. After temperature equilibrium TEER was determined in order to prove no occurrence of adverse effects on tight junction function due to test solution application.

After transport studies total fluorescence of APTS-dextrans of the samples were determined using a plate reader, followed by CE as described above. Thus, percentages of total fluorescence for every single peak were determined and clearance values were calculated with Eq. (1) for cultured membrane inserts and blind values.

$$\text{clearance} = \frac{C_{B_n} V_B}{C_A - \left(\frac{V_B}{V_A} \sum C_{B_{n-1}}\right)} \quad (1)$$

C_{B_n} is the substance concentration into the basolateral chamber and C_A is the concentration into the apical compartment. V_A and V_B are the according volumes of the chambers. Since the amount of the compound in the apical insert will decrease over time under conditions of unidirectional flux, C_A value for each time point (each well) has to be corrected. Therefore, the summed up absolute amount of each substance found in the basolateral compartment before the actual one ($\sum C_{B_{n-1}}$) is related to the apical volume (V_B/V_A) and substracted from C_A. For calculating permeability coefficient values, slope of clearance versus time is determined by linear regression analysis and multiplied with a factor considering the growth surface area (4.2 cm^2). Finally, the reciprocal value of the permeability coefficient without cell layer (PE_{blank}) is substracted of the permeability coefficient of the experiment with cell layer (PE_{all}) to gain the reciprocal value only for the cell layer (Eq. (2)).

$$\frac{1}{PE_{cell}} = \frac{1}{PE_{all}} - \frac{1}{PE_{blank}} \quad (2)$$

For analysis of diazepam RP-LC was used and permeability coefficients were determined by same procedure.

3. Results and discussion

In Fig. 2 a typical electropherogram for commercially available FD4 (Sigma) in C6-medium is depicted. A single peak of FITC and a broad hill of FD4 is recognisable, which represents the distribution of an unseparated mixture of dextrans. These electropherograms were just useful for correcting permeability coefficients of FDs considering the free FITC amount. Further work was concentrated on separation of mixtures of several purchasable FDs with different average molecular weights (FD4, FD10S, FD20 and FD40, Sigma). This approach did not lead to effective results. Also in house labeling of dextrans with FITC was not succussful in obtaining a satisfying analytical method. Separation by CE was significantly complicated due to the labeling strategy, because FITC binds to hydroxyl groups of dextran in a statistical manner. This led to the search for a strategy that would allow a 1:1 molar ratio of dextran to fluorescence marker. Based on previous experience [15–17], it was decided to use the terminal carbonyl moiety of the dextran molecules, which reacts easily in reductive amination. APTS was chosen because of its comparable fluorescent properties to FITC. Fig. 3 shows a electropherogram of APTS-labeled dextran 6000. Following the strategy of specific reductive amination, separation was remarkably improved. After labeling, each dextran fraction was linked to one APTS molecule giving all products the

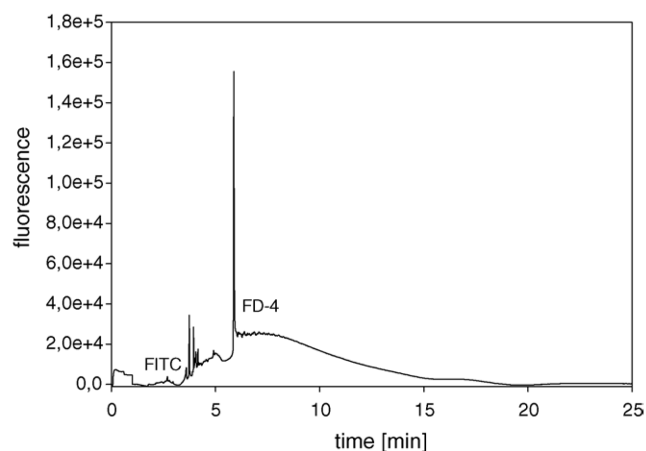

Fig. 2. Typical electropherogram of FD4 in C6-medium. It is not able to differentiate between several fractions besides the early FITC peak.

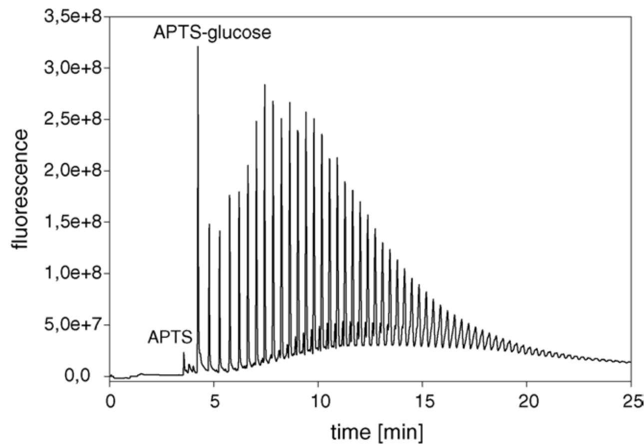

Fig. 3. Electropherogram of APTS-labeled dextran 6000 in C6-medium. In comparison to FD4 every single peak of APTS-dextran 6000 is analyzable. Molecular size of each fraction is increased about one glucose unit compared to its left neighbouring peak.

same charge. Consequently, migration through electrical field was only dependent on molecular size and weight using PEO as polymeric additive. Suzuki et al. [18] have confirmed with MALDI-MS measurements that every fraction differs from the neighboured one in the electropherogram about one glucose unit. Solutions of 240 μM APTS-labeled dextran 6000 in C6-medium were used for transport studies as described before. Firstly, total fluorescence amounts of samples were measured using a plate reader, afterwards percentage of each dextran fraction was determined by CE. Using these data, permeability coefficients were calculated. For experiments with APTS-dextran 6000 confluent cell layers with TEER values of $100 \pm 8.4\ \Omega\ \mathrm{cm}^2$ were used. Permeability coefficient of total dextran was 9.17 ± 0.45 μm/min ($n = 3$). Detailed results of single dextran fractions are listed in Table 1. In order to demonstrate the influence of the coated membrane (PE_{blank}), effect of correction (%) is presented additionally. The obtained permeability pattern of APTS-labeled dextran 6000 through the PBMEC/C1-2 layer is depicted in Fig. 4. As expected, a molecular size dependent permeability was observed. Our concept using the APTS-labeled dextran ladder for description of paracellular transport is based on the

Table 1
Summarized calculated PE values of blank and cell experiments with APTS-dextran 6000

Number of glucose units	PE_{blank} (μm/min)	PE_{all} (μm/min)	PE_{cell} (μm/min)	Effect of correction (%)
0	43.09 ± 0.42	12.59 ± 1.40	16.38 ± 3.79	130.12
1	18.65 ± 1.66	7.77 ± 0.17	13.30 ± 0.65	171.23
2	18.94 ± 1.15	6.67 ± 0.25	10.46 ± 0.56	156.87
3	19.55 ± 1.02	7.18 ± 0.20	10.94 ± 0.48	152.50
4	18.13 ± 0.50	6.48 ± 0.09	10.25 ± 0.24	158.18
5	18.81 ± 0.52	6.76 ± 0.11	10.77 ± 0.15	159.31
6	17.43 ± 0.69	6.42 ± 0.08	10.33 ± 0.24	160.82
7	17.10 ± 0.84	6.34 ± 0.17	10.38 ± 0.03	163.68
8	16.59 ± 0.43	6.31 ± 0.12	10.31 ± 0.23	163.35
9	16.61 ± 0.60	5.66 ± 0.16	8.85 ± 0.04	156.23
10	16.69 ± 0.43	5.59 ± 0.23	8.66 ± 0.30	154.90
11	16.55 ± 0.33	5.28 ± 0.14	8.00 ± 0.11	151.53
12	16.09 ± 0.25	5.42 ± 0.18	8.46 ± 0.00	156.10
13	15.88 ± 0.23	5.09 ± 0.19	7.82 ± 0.10	153.45
14	15.75 ± 0.40	5.09 ± 0.19	7.87 ± 0.33	154.58
15	15.36 ± 0.30	4.95 ± 0.28	7.71 ± 0.11	155.68
16	14.85 ± 0.40	5.08 ± 0.13	7.85 ± 0.20	154.46
17	14.55 ± 0.27	4.84 ± 0.16	7.48 ± 0.06	154.56
18	14.25 ± 0.24	4.72 ± 0.25	7.49 ± 0.10	158.76
19	13.88 ± 0.23	4.70 ± 0.15	7.39 ± 0.41	157.07
20	13.87 ± 0.23	4.54 ± 0.34	7.10 ± 0.51	156.28
21	13.56 ± 0.40	4.71 ± 0.42	7.45 ± 1.07	158.12
22	13.60 ± 0.60	4.52 ± 0.31	7.34 ± 0.23	162.35
23	13.23 ± 0.51	4.61 ± 0.38	7.73 ± 0.02	167.80
24	13.12 ± 1.03	4.52 ± 0.07	6.92 ± 0.16	153.21
25	13.61 ± 1.05	4.47 ± 0.52	6.81 ± 1.40	152.18
26	13.94 ± 1.15	4.67 ± 0.31	7.60 ± 0.71	162.81
27	14.23 ± 0.72	4.41 ± 0.34	6.26 ± 1.01	141.82
28	14.24 ± 1.69	4.68 ± 0.52	7.84 ± 0.10	167.63
29	13.97 ± 2.09	4.60 ± 0.34	6.39 ± 0.95	139.07
30	14.98 ± 1.44	5.07 ± 0.14	7.45 ± 0.03	146.91
31	16.54 ± 1.39	4.76 ± 0.51	6.07 ± 1.23	127.38

PE values of single APTS-dextran 6000 fractions are presented as the means ± S.D. of blank (PE_{blank}, $n = 3$) and cell experiments (PE_{all}, $n = 3$). Number of glucose units = 0 means APTS. Test solutions consisted of 240 μM APTS-labeled dextran 6000 added with 50 μM diazepam as internal standard in C6-medium. Effect of correction (%) means percentage of PE_{cell} values related to PE_{all} values, which is the total permeability through the cell layer, the coating and finally through the membrane of the insert.

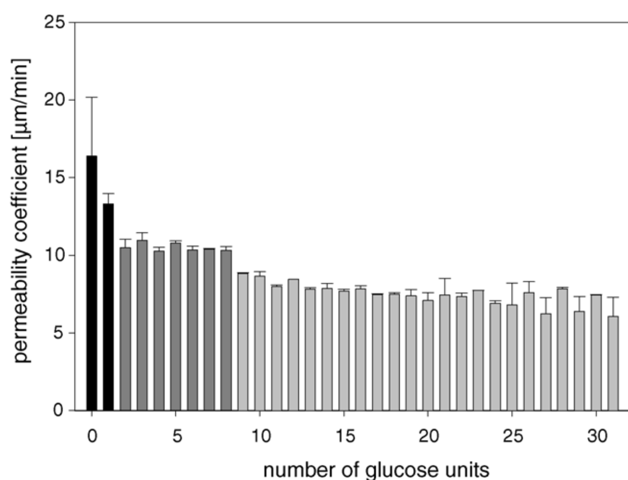

Fig. 4. Permeability pattern of transport studies with APTS-dextran 6000 with an established in vitro BBB transwell model culturing immortalized endothelial cell line PBMEC/C1-2. Permeability coefficients (PE_{cell}) (μm/min) were depicted against the number of glucose units, of which each fraction consisted. Number of each experiment was 3. Number of glucose units = 0 means APTS.

structural properties. Each molecule is very hydrophilic and carries three negatively charged sulfonic acid groups. Therefore, passive transcellular diffusion is improbable.

Permeability of the internal standard diazepam (mean ± S.D.: 67.97 ± 7.32 μm/min, $n = 3$) represented transcellular transport and was significantly higher than total permeability of paracellularly transported APTS-dextran. The diazepam results were comparable to other transport studies with this BBB transwell model.

After transport experiments TEER measurements of both cell monolayers in apical APTS-dextran solution as well as after supernatant removal and temperature equibrilium in growth medium indicated no break down of tight junctions. Additionally, no cell detachment was detected microscopically. Thus, the test solution had no negative influence on cell viability and was not celltoxic.

In comparison to commonly used FDs, the APTS-labeled dextran permeability pattern is able to reflect paracellular transport in drug relevant size ranges and to monitor its changes over time, whereas FD studies result in average permeability values without differentiating between the applied fluorescent fractions. Furthermore, purchaseable FDs contain amounts of non-labeled dextrans, e.g. in FD4 (average molecular weight = 4000 g/mol) only one dextran out of 10 is statistically labeled with FITC. Thus, real permeated dextran amount is about ten times higher. Nevertheless, for general tightness determination and detecting significant changes using FDs is sufficient and well established, because calculation methods for transwell transport studies relate permeated amounts to the stock solution and thus only includes fluorescent labeled dextrans.

4. Conclusion

The introduced technique for characterization of cell layer tightness enables to display permeability patterns depending on molecular size. In comparison to other currently used methods, the APTS-dextran ladder is more precise in describing paracellular transport pathways. It could be applied for determining tightness status and integrity of cell layers, especially for the BBB. Further studies will focus on describing cell layers with higher TEER like Caco-2 or ECV304, in order to be able to relate permeability patterns to TEER values. In conclusion, the APTS-dextran ladder is an useful tool for characterizing cell layer tightness applying a wide range from smaller to larger molecules at the same time in order to refine, e.g. TEER, sucrose or Evans blue results.

Acknowledgements

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