A hydrogel-based *in vitro* assay for the fast prediction of antibiotic accumulation in Gram-negative bacteria

Robert Richtera,b, Mohamed A. M. Kamala, Mariel A. García-Riverac, Jerome Kaspar,d, Maximilian Junkd, Walid A. M. Elgaherea, Sanjay Kumar Srikakulam1, Alexander Gress1, Anja Beckmanng, Alexander Grißmereg, Carola Meierg, Michael Vielhaberd, Olga Kalininafh, Anna K. H. Hirscb,b, Rolf W. Hartmanne, Mark Brönstruptcf,i, Nicole Schneider-Dauma and Claus-Michael Lehmab,b,*

a Department of Drug Delivery, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infection Research (HZI), 66123 Saarbrücken, Germany, E-mail: Claus-Michael.Lehr@helmholtz-hips.de

b Department of Pharmacy, Saarland University, 66123 Saarbrücken, Germany

c Department of Chemical Biology, Helmholtz Centre for Infection Research (HZI), 38124 Braunschweig, Germany

d Institute of Engineering Design, Saarland University, 66123 Saarbrücken, Germany

e Department of Drug Design and Optimization, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infection Research (HZI), 66123 Saarbrücken, Germany

f Department of Drug Bioinformatics, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz, Centre for Infection Research (HZI), 66123 Saarbrücken, Germany

g Department of Anatomy and Cell Biology, Saarland University, 66421 Homburg, Germany

h Medical Faculty, Saarland University, 66421 Homburg, Germany

i German Centre for Infection Research (DZIF), 38124 Braunschweig, Germany

* Claus-Michael Lehr, Department of Drug Delivery, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Campus E8.1, 66123 Saarbruecken, Germany

Email: Claus-Michael.Lehr@helmholtz-hips.de

Phone: +49681988061000


### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| %IncMSE      | relative increase of mean squared error in % |
| AMP          | ampicillin |
| AZT          | aztreonam |
| CEF          | cefuroxime |
| CHL          | chloramphenicol |
| CIP          | ciprofloxacin |
| CLI          | clindamycin |
| cdogD$_{pH7.4}$ | calculated common logarithm of distribution coefficient at pH 7.4 |
| CLSM         | confocal laser scanning microscopy |
| ERY          | erythromycin |
| FUS          | fusidic acid |
| HPLC         | high performance liquid chromatography |
| IMI          | imipenem |
| KAN          | kanamycin |
| LC           | liquid chromatography |
| LEV          | levofloxacin |
| LIN          | lincomycin |
| magn.        | magnification |
| MIC          | minimum inhibitory concentration |
| MIN          | minocycline |
| NOR          | norfloxacin |
| NOV          | novobiocin |
| NAL          | nalidixic acid |
| Omp          | outer membrane protein |
Abstract

The pipeline of antibiotics has been for decades on an alarmingly low level. Considering the steadily emerging antibiotic resistance, novel tools are needed for early and easy identification of effective anti-infective compounds. In Gram-negative bacteria, the uptake of anti-infectives is especially limited. We here present a surprisingly simple in vitro model of the Gram-negative bacterial envelope, based on 20% (w/v) potato starch, printed on polycarbonate 96-well filter membranes. Rapid permeability measurements across this polysaccharide hydrogel allowed to correctly predict either high or low accumulation for all 16 tested anti-infectives in living E. coli. Freeze-fracture TEM supports that the macromolecular network structure of the starch
hydrogel may represent a useful surrogate of the Gram-negative bacterial envelope. Machine learning by random forest analysis of \textit{in vitro} data revealed minimum projection area, molecular mass, and rigidity as the most critical physicochemical parameters for hydrogel permeability, in agreement with reported structural features needed for uptake into Gram-negative bacteria. Correlating our data set of 27 antibiotics from different structural classes to reported MIC values of four clinically relevant pathogens allowed to distinguish active from non-active compounds based on their low \textit{in vitro} permeability and in particular to identify poorly permeable antimicrobial candidates before testing them on living bacteria.

\textbf{Keywords}

Structure–permeability relationships, antibiotic screening, starch hydrogel, printing, random-forest analysis

1. Introduction

Since their “Golden Age” (1930’s to 60’s), the number of novel antibiotics has been steadily decreasing [1,2], while bacterial resistance is continuously increasing [3]. Especially, infections caused by Gram-negative bacteria are about to lack appropriate treatment, because the pipeline of new anti-infective compounds is only poorly filled [4]. While the limited profitability of antibiotic research is one crucial factor for this crisis, the development of novel antibiotics is additionally hampered by intrinsic and acquired resistance mechanisms of bacteria [5]. Since most of the antibiotics used against Gram-negative infections, such as β-lactams, tetracyclines, fluoroquinolones and aminoglycosides have to reach intracellular targets; it becomes obvious that the Gram-negative bacterial cell envelope is an important hurdle. The elements of this biological barrier (e.g., outer membrane proteins, lipopolysaccharides, periplasmic space, β-lactamases, cytoplasmic membrane) and their efficacy in limiting the uptake of antibiotics have already been described previously [6–13]. Consequently, the need emerged to find suitable methods to evaluate and predict the permeability of anti-infectives. These attempts include \textit{in vitro} approaches such as liposome [14] or outer-membrane-vesicle swelling assays [15], electrophysiological studies [16–18], as well as assays employing lipid-coated filters [19,20], reconstituted bacterial vesicles [21] or
giant unilamellar vesicles (GUV’s) [22]. Further, important assays have been
developed using living bacteria [23–26]. Moreover, several in silico simulations of drug
transport across porins exist [27–30]. However, the currently available methods are of
limited accuracy, versatility and work-place safety, are time-consuming and suffer from
relatively high costs with at the same time low-throughput capacity [31]. Here, we
present a high-throughput concept for a relatively simple, quick and cost-effective
assay to predict compound permeability across the Gram-negative bacterial cell
envelope and thus bacterial uptake. In spite of its simplicity, it shows surprisingly high
accuracy and predictability, qualifying it as a useful tool for optimizing the passive
transport of anti-infective compounds across this important biological barrier and for
excluding many inactive compounds already at an early stage of antimicrobial drug
development.

2. Materials and methods

2.1 Materials

MultiScreen® 96-well Filter plates with 0.4 µm PCTE membrane and MultiScreen® 96-
well Transport Receiver Plates were obtained from EMD Millipore Corporation
(Billerica, Ma, USA). Sodium alginate (Protanal LF 10/60 FT) was obtained from FMC
Biopolymer UK Ltd. (Girvan, Ayrshire, UK). Amylopectin (ELIANE 100) and potato
starch (Partially hydrolysed, M_w > 1.500 kDa, amylose content 33%) were donated by
AVEBE U.A. (Veendam, NE). Agarose SERVA (research grade) and Streptomycin-
SO_4 were obtained from SERVA Electrophoresis GmbH (Heidelberg, Germany).
Ampicillin-Na (CELLPURE®) was obtained from Carl Roth GmbH + Co. KG (Karlsruhe,
Germany). Aztreonam was obtained from MP Biomedicals, LLC (Illkirch, France).
Tetracycline-HCl was obtained from chemodex (St. Gallen, Switzerland). Rifampicin
was obtained from USBiological (Swampscott, MA, USA). Tigecycline and pipemidic
acid were obtained from LKT Laboratories, Inc. (St. Paul, MN, USA). Imipenem was
obtained from MOLEKULA® GmbH (Munich, Germany). Amylose, novobiocin sodium
and sulfamethoxazole were purchased from Cayman Chemical Company (Ann Arbor,
MI, USA). Phosphate buffered saline (PBS, pH 7.4) was prepared from dissolution of
0.02 M PBS tablets without potassium (Genaxxon Bioscience, Ulm, Germany) in 1 L
of Milli-Q water. Hydrochloric acid and sodium hydroxide solutions (1 M each) were
used from Bernd Kraft (Duisburg, Germany). Methanol, acetonitrile (both HPLC grade),
acetic acid (glacial) were obtained from VWR Chemicals (VWR International S.A.S., Fontenay-sous-Bois, France). Methylene blue was obtained from J.T. Baker (Avantor™ Performance Materials, Radnor, PA, USA). Fluoraldehyde™ (o-phthalaldehyde reagent solution) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Chitosan (high molecular mass), tobramycin, kanamycin monosulfate, phosphomycin disodium, erythromycin, ciprofloxacin, chloramphenicol, norfloxacin, minocycline hydrochloride, sparflaxacin, fusidic acid sodium, levofloxacin, clindamycin hydrochloride, lincomycin hydrochloride, cefuroxime sodium were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2 Methods

Membrane preparation. Due to differences in the viscoelastic properties of the employed polysaccharide gels, concentrations varied between the different polysaccharides. Alginate gels of 5, 10, 15 and 20% (w/v) were made by suspending respective amounts of Protanal LF 10/60 FT in 30 mL of Milli-Q water. The suspension was kept overnight at a 70 °C water bath to allow for complete dissolution. For 3% (w/v) chitosan gel 300 mg high molecular mass chitosan were dissolved overnight in 10 mL acetic acid (1% v/v). 0.02 M sodium hydroxide solution was optionally given for neutralization (pH 7.4). For 2.5, 5, 7.5, 10% (w/v) agarose gels, the respective amount of agarose was suspended in 10 mL Milli-Q water and heated in the microwave for 1 min at 600 W. For 10, 15, 20, 25, 30, 35% (w/v) starch gels; 10, 15, 20 and 25% (w/v) amylopectin, and 30, 40, 50, 60% (w/v) amylose, slightly acid degraded potato starch, amylopectin or amylose, respectively, were suspended in 10 mL of Milli-Q water and boiled until a clear solution formed. A displacement pipette (Transferpettor®, Brand GmbH & CoKG, Wertheim, Germany) was used to coat each filter support of MultiScreen® 96-well Filter plates with 40 μL of the respective polysaccharide formulation. The covered and coated filter plates were kept overnight at 4 °C.

Preparation of donor solutions. 100 μg/mL Donor solutions of rifampicin, novobiocin, tetracycline, clindamycin and chloramphenicol were prepared by direct dissolution of the compounds in PBS (pH 7.4). 100 μg/mL solutions of quinolones and nalidixic acid were prepared by dissolving 1 mg of compound in 1 mL of 0.1 M sodium hydroxide solution, adding 4 mL of PBS (pH 7.4), neutralizing the solution with 1 M hydrochloric acid and filling up to 10 mL by PBS (pH 7.4).
**In vitro permeability assay.** Assays were performed using the gel coated donor wells of 96-well filter plates in combination with a 96-well receiver plate. After equilibration of the gel coatings from both sides in PBS (pH 7.4) at 37°C for 30 min, PBS was removed and 230 µL pre-warmed antibiotic donor solution (37 °C) were given into the respective donor wells, while 30 µL were immediately removed and diluted 1:10 in an extra plate. 300 µL of fresh PBS (pH 7.4) were given into the corresponding wells of the receiver plate. Donor and acceptor plate were reassembled, sealed with adhesive foil, and incubated (37 °C, 180 rpm). At 0, 10, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240 min, the transport system was disassembled to measure the absorbance in the acceptor wells of the receiver plate using a Tecan Infinite® 200 PRO (Tecan Trading AG, Maennedorf, Switzerland) plate reader. An adjusted protocol was followed for substances with insufficient absorbance. In case of tobramycin and kanamycin, samples of 20 µL were drawn and a quick fluorimetric approach was followed by diluting samples 1:11 by Fluoraldehyde™. The removed volume was replaced using fresh PBS (pH 7.4). In the case of other substances with insufficient absorbance signal, 220 µL of donor solution were given in each donor well. 20 µL were immediately removed and diluted 1:10. At all time points, samples of 40 µL were drawn from acceptor wells and diluted 1:5 for liquid chromatography coupled mass spectrometry (LC–MS). Permeated amounts of each compound were calculated in reference to calibration curves, which were prepared from the applied donor solution.

Quantification by UV-spectroscopy. At selected time points, the antibiotic concentration in the receiver plates was directly quantified using a Tecan Infinite® 200 PRO plate reader, run by Tecan i-control, 1.10.4.0 software (Tecan Trading AG, Maennedorf, Switzerland). Antibiotics and absorbance wavelengths used for their quantification are listed in Table S1.

Quantification by fluorimetry. After reaction with Fluoraldehyde™, fluorescence was measured (λ_{ex} = 360 nm, λ_{em} = 470 nm) using a Tecan Infinite® 200 PRO plate reader and Tecan i-control, 1.10.4.0 software (both Tecan Trading AG).

Quantification by LC–MS/MS. An Accela UHPLC system coupled TSQ Quantum Access Max tandem quadrupole mass spectrometer (both from Thermo Fisher Scientific, Waltham, MA, USA) was used. The UHPLC device featured a quaternary mixing pump, an online degasser, and a column oven. The entire system was operated via the standard software Xcalibur™ (Thermo Fisher Scientific, Waltham, MA, USA). Streptomycin samples were quantified using a Synchronis HILIC column (50 × 2.1 mm,
1.7 µm, Thermo Fisher Scientific) column. For all other compounds, an Accucore RP-MS column (150 × 2.1, 2.6 µm, Thermo Fisher Scientific, Waltham, MA, USA) was employed. The chromatographic analysis was performed with a binary solvent mixture using optionally acetonitrile + 0.1% formic acid (A), MilliQ-Water + 0.1% formic acid (B), methanol + 0.1% formic acid (C) or ammonium formate buffer (10 mM, pH 3, D). Ampicillin and sulfamethoxazole were analysed using an isocratic run with 60% B and 40% C or 40% A and 60% B, respectively. All other compounds followed a gradient run. As for clindamycin and lincomycin, the initial value of 18% A and 72% D was shifted to 30% A and 70% D within 2 min and then kept constant for another 2 min. As for tobramycin and streptomycin, the ratio of 95% A and 5% B was shifted after 2 min to 5% A and 95% B within 1.5 min, and then was kept constant for 3.5 min. Vancomycin samples were run for the first minute with 5% A and 95% B before shifting within 1 minute to 95% A and 5% B and keeping the values for 3 min. Erythromycin was run starting with an immediate shift from 18% A and 82% D to 90% A and 10% D within 2 min. The latter ratio was kept constant for 3 min. Fusidic acid started with 35% B and 65% C. After 2 min, the values changed to 5% B and 95% C within 1 min. After that, these values were kept constant for 4 min. The detection of the compounds in the MS happened after heated electrospray ionization (H-ESI) during positive ion mode using for sulfamethoxazole single ion monitoring (SIM) and for all others selective reaction monitoring (SRM). LC–MS/MS parameters are summarized in Table S2.

**Calculation of the apparent permeability coefficient (P\textsubscript{app}).** A regression at the linear slope, at which the accumulated drug amount has not yet exceeded 10%, yields the rate of compound flux (J). P\textsubscript{app} is then calculated using the following formula:

\[
P_{\text{app}}(cm \ast s^{-1}) = \frac{J}{A \ast c_0}
\]

Where \(c_0\) is the initial donor concentration (µg/cm\(^3\)), \(A\) is the surface area of the filter support (cm\(^2\)) and \(J\) is the compound flux (µg/cm\(^3\)).

**Validation of starch-based permeability assay.** Transport studies carried out for assay validation, were performed under a reduced number of time points (10, 20, 30, 45, 60 and 90 min) and antibiotic donor solutions had a concentration of 200 µM. Since
not all curves showed linear permeation behaviour between 10 and 30 min, the area under the curve (AUC\textsubscript{10-30 min}) was calculated by integrating the permeation-time curves within the limits of 10 to 30 min.

**Application of starch-based permeability assay on RNAP-inhibitors.** Permeation studies on previously reported in-house RNAP inhibitors 1–3 [32] were carried out as mentioned under ‘in vitro permeability assay’. The absorbance in the receiver plate was measured at time points 10, 20, 30, 45, 60 and 90 min. The initial donor concentration was 100 µM for each compound.

**Printing of membranes.** A customized 3D-printer was designed based on modular aluminium construction profiles with controls based on a Duet 2 32 Bit 3D-printer controller running a customized version of RepRapFirmware 2.02. The printer features igus\textsuperscript{®} SHT spindle drive linear axes fitted with igus\textsuperscript{®} MOT-AN-S-060-020-056-L-A-AAAAA motors (both igus\textsuperscript{®} GmbH, Cologne, Germany) for x/y/z motion with a resolution of 5 µm ± 5% and theoretical microstepped resolution of up to 0.625 µm. Extrusion of the starch solution is accomplished by a 10 mL Hamilton\textsuperscript{®} SaltLine reagent syringe (model 1010 TLL-SAL, Hamilton\textsuperscript{®} Company, Reno, NV, USA) driven by a Nanotec L4118S1404-M6X1 Hybrid linear actuator (Nanotec Electronic GmbH & Co. KG, Feldkirchen, Germany; full-step volume resolution of the driven syringe 0.837 µL per step). The syringe was kept at 80 °C by a VWR\textsuperscript{®} refrigerated circulating bath with a digital temperature controller (model 1166D, VWR\textsuperscript{®} International, LLC., Radnor, PA, USA). The hot water supply for the syringe consisted of a Masterflex L/S\textsuperscript{®} easy-load\textsuperscript{®} peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA) adjusted to speed 1 and OMNILAB silicone tubing (5 × 2 × 9 mm, OMNILAB-LABORZENTRUM GmbH & Co. KG, Bremen, Germany) as well as PhthalateFree\textsuperscript{®} PVC Pump Tubes (3.18 mm ID, Gilson\textsuperscript{®} Company Inc., Lewis Center, OH, USA). A trimmed Sterican\textsuperscript{®} needle for special indications (G 14 × 3 1/8" / Ø 2.10 × 80 mm, B. Braun Melsungen AG, Melsungen, Germany) was used as a nozzle. The g-code used to print the starch solution into the donor wells can be found in the supplementary information.

**Absorbance scan of membranes and quantitative structural comparisons.** As a quick check for batch homogeneity and evenness, 20% (w/v) starch solutions were prepared in 100 µg/mL methylene blue solution before coating the filter membrane. After coating filter plates underwent an absorbance analysis using the Tecan Infinite\textsuperscript{®}
200 PRO platereader, with Tecan i-control, 1.10.4.0 software (Tecan Trading AG, Maennedorf, Switzerland). The scan was done at 666 nm (bandwidth 9 nm), using 15 × 15 reads/well with 25 flashes/read, no settle time and a border of 850 nm. As plate type, ‘Millipore MultiScreen 96 Flat Bottom Transparent Polysterol’ was selected. The obtained absorbance values per well were aligned in x and y direction and subsequently plotted as a 3D surface diagram using Microsoft® Excel® for Microsoft 365 MSO (16.0.12827.20200), Microsoft® Corp., Redmond, WA, USA).

Quantitative assessment of gel coating. Gel deposition was evaluated by calculating the average absorbance per membrane using the absorbance data of 177 points of nine wells and calculating the overall mean. The intra-well-variability is represented by the mean standard deviation of all performed gel coatings. Regarding the intra-batch-variability, the standard deviation of the mean absorbance of each coated well was averaged for each batch before calculating the mean of the obtained values. The inter-batch-variability was obtained by calculating the standard deviation of the mean absorbance from each batch. Obtained data originate from at least 3 batches of membranes consisting of at least triplicates.

Transmission electron microscopy (TEM) of freeze-fracture replicas. For the analysis of the polysaccharide gels in TEM, starch hydrogels were prepared by dissolving modified potato starch in water (final concentrations were 10% or 20% or 40% (w/v)). Hydrogels of 10% agarose, 20% amylopectin and 30% amylose (w/v) were prepared in water too. The solutions were dropped onto glass cover slips and kept in the refrigerator for solidification. 24 hours later, small slices of the hardened hydrogel were cut with a scalpel. The slices were incubated with phosphate-buffered saline (PBS) for 30 min at 37 °C and 5% CO₂. Thereafter, small pieces of the hydrogel slices were mounted in between a sandwich of copper carriers (one flat-bottom, one with depression) and plunge-frozen into a nitrogen-cooled liquid ethane-propane mixture using the Leica plunge freezer EM CPC (Leica Microsystems, Wetzlar, Germany).

Afterwards, the sandwich carriers were mounted in a cryo-preparation box onto a nitrogen-cooled finger replica table and transferred with an EM VCT shuttle into the EM BAF060 freeze-fracture and etching device (all devices from Leica Microsystems). Freeze-facturing was performed at -162 °C and 1 × 10⁻⁷ mbar by chipping off the upper copper carrier. Fractured samples were etched for 5 min at -100 °C. The etched faces were rotary shadowed with a 1.5 nm platinum-carbon coating applied at a 60 ° angle,
followed by a 20 nm carbon coat applied at 90 °. The replicas were stabilized on a gold index grid using 0.5% Lexan polycarbonate plastic dissolved in dichloroethane (DCE).

After evaporating the DCE by incubating the sample-replica-Lexan-grid at -20 °C for 16 h, the samples were thawed at room temperature and the carriers were removed. Grids were given into 70% sulfuric acid for 3 h to dissolve the starch from the replicas. Afterwards, grids were given into double-distilled water for 1 h. The grids were dried on filter sheets and a 20 nm carbon coating was applied at 90° for further stabilization.

After removal of the Lexan film by incubation in hot DCE, analysis was performed using a FEI Technai G2 transmission electron microscope (FEI, Thermo-Fisher Scientific, Munich, Germany) at 100 kV, equipped with a digital 8-bit camera. The negative contrast was reversed for image interpretation, so that the heavy metal appears white and the shadow appears black.

**In bacterio control assay.**

**Bacterial uptake.** 5 mL of lysogeny broth (LB) were inoculated with *E. coli* MG1655 and incubated overnight at 37 °C and 150 rpm. 2 × 60 mL of fresh LB were inoculated with 1 mL of overnight culture (starting OD₆₀₀ ≈ 0.1) and incubated (37 °C, 150 rpm) till reaching OD₆₀₀ = 0.7. The bacterial culture was centrifuged in 50 mL Falcon tubes (9 min, 4500 x g, 20 °C), the supernatant was removed and following resuspension of the pellet in 5 mL NaPi-MgCl₂ buffer (50 mM sodium phosphate (NaPi) + 5 mM MgCl₂ adjusted to pH 7.0, sterile filtrated) the suspension was centrifuged under the same conditions. Again, the supernatant was discarded, and the pelleted resuspended in warm NaPi buffer to reach OD₆₀₀ = 5.0. The obtained suspension was kept at 37 °C for 5 min. 100 µL/well of bacterial suspension were given into a MultiScreenHTS DV filter plate (transparent, pore size 0.45 µm, Merck Millipore, Tullagreen, IRL) wetted with 2 µL NaPi buffer. At time points 0, 2, 5, 10, 20, 30, 42, 47, 50, 52 min, 25 µL of the respective antibiotic solution was added and mixed in the corresponding wells to give a final concentration of 200 µM. The filter plate was shaken at 350 rpm and 37 °C in a ThermoMixer® C (Eppendorf GmbH, Hamburg, Germany) during antibiotic addition. For the 0 min time point, 25 µL of antibiotic solution were added right before filtration. The incubation was stopped at the respective time point by removal of the supernatant with a vacuum manifold (~15 s) and washing the cells twice with 100 µL of ice-cold NaPi buffer by a Bravo Automated Liquid-Handling Platform (Agilent Technologies, Santa Clara, CA, USA) and filtered again. The filter plate was pressed against
absorbent paper to remove the remaining liquid after every filtration. The filter plate was put on top of a 350 µL conical bottom receiver plate (clear polypropylene, Greiner Bio-One GmbH, Frickenhausen, Germany) and pellets were resuspended in 100 µL of ice-cold methanol-water blend (8:2). After that, the suspension was incubated for 30 min at RT and 400 rpm while being sealed with Parafilm® (Bemis Company Inc., Neenah, WI, USA) and closed with a lid. Following the incubation step, the filter plate was centrifuged at 2250 × g for 5 min and the filtrate collected in the receiver plate. The cell debris was further lysed by adding 100 µL of ice-cold acetonitrile to the filtrate and mixing before it was incubated for 30 min at RT and 400 rpm. Further centrifugation at 2250 × g for 15 min and collection of filtrate was then followed by evaporation using a centrifugal vacuum concentrator at 20 °C coupled to a cold trap at -50 °C (both from Labconco Corporation, Kansas, MO, USA). The dry remnants were reconstituted in 100 µL of a methanol-acetonitrile blend containing 0.1% formic acid and 10 ng/mL caffeine as internal standard, with the exception of streptomycin and tobramycin, which were reconstituted in 100 µL of water containing 0.1% formic acid and 10 ng/mL caffeine. Samples were subsequently measured by LC–MS/MS (see below). To determine the unspecific binding of the tested compounds, 100 µL of NaPi buffer were added to a blank filter plate and incubated for 5 min at 37 °C. 25 µL of compound from stock solutions were added and mixed as mentioned before. The plate was incubated until the 52 min time point and, from then on, treated like the bacteria-containing plate. The amount of antibiotic in bacterial samples was determined based on a corresponding regression curve. To calculate the effective accumulated amount obtained, the amount of corresponding compound in nmol from unspecific binding was subtracted from all data obtained from bacterial incubation.

Quantification of uptake by LC–MS/MS. Samples were quantified by an Agilent 1290 UHPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to an AB Sciex QTrap 6500 ESI-QQQ (AB Sciex Germany GmbH, Darmstadt, Germany) mass spectrometer. For chromatographic separation, a ZORBAX Eclipse Plus C18 (2.1 × 5.0, 1.8 µm, Agilent Technologies, Santa Clara, CA, USA) column was employed. A linear gradient was applied using water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), in which the initial amount 99% A shifted to 1% A over a period of 5 min and at a flow rate of 0.7 mL/min. Chromatographic separation of tobramycin samples was carried out in a Shodex HILICpak VC-50 2D column (20 × 150 mm, 5 µm, Showa Denko America Inc., NY, USA). A linear gradient was applied using water + 1.5% ammonia
(A) + and acetonitrile 0.1% formic acid, starting at 70% A and reaching 90% A over a period of 5 min and at a flow rate of 0.3 mL/min. LC–MS/MS parameters are summarized in Table S3.

Random forest analysis. The random forest model was created using randomforest library [33] in R (v. 3.6.1; R Core Team, R Foundation for Statistical Computing; 2017, Vienna, Austria). The input data (AUC10-30 min, net charge, molecular mass, minimum projection area, relative abundance of unsaturated bonds, number of rotatable bonds, number of hydrogen bond acceptors, number of hydrogen bond donors, globularity, clogD_{pH7.4}) was initially processed and stored as a table. We used leave-one-out cross validation to develop this model with the hyperparameters ntree of 100, mtry of 2 and maxnodes of 8. Where ntree is the number of decision trees we allow this model to grow, mtry is the number of variables randomly sampled at each split or tree node and maxnodes is the maximum number of terminal nodes every tree in the forest can have. The depth of the tree can be controlled using the maxnodes hyperparameter. In the process of model optimization, clogD_{pH7.4} and number of rotatable bonds were removed as input parameters to enhance robustness and avoid overprediction. To ensure reproducibility, the seed value '6' was randomly selected. All physicochemical parameters were calculated by Molecular Operating Environment (MOE) 2019.0102 (relative abundance of unsaturated bonds, number of rotatable bonds, H bond acceptors and donors, globularity) and chemicalize.com (net charge, molecular mass, minimum projection area, clogD_{pH7.4}). The code used for the analysis is enclosed in the supplementary information. Physicochemical properties are available in Dataset S1.

Calculation of metric molecular sizes. The approximate size of ciprofloxacin and novobiocin in nm was determined manually using the Visual Molecular Dynamics (VMD) program, version 1.9.4. [34]

Significance tests/Plots. Tests for significance and plotting of permeation data were carried out using GraphPad Prism® 7.04 software (GraphPad Prism software Inc., San Diego, CA, USA).
3. Results and discussion

3.1 Selection of polysaccharide gel

Antibiotics with intrabacterial targets have to pass through numerous barriers (Figure 1) and are potentially subjected to enzymatic degradation and efflux [35]. It is reported that for small hydrophilic antibiotics, porins play a key role in uptake [6,36] and may even govern the overall compound accumulation inside bacteria [37].

Figure 1. Permeation barriers of Gram-negative bacteria. (a) Biofilm (optional), (b) lipopolysaccharide-rich outer leaflet of outer membrane, (c) phospholipid-rich inner leaflet of the outer membrane, (d) porin, (e) multidrug efflux pump, (f) periplasmic space, (g) Plasma membrane.
Hence, this route is obviously of major significance for designing a predictive permeation model for small antibiotic molecules. Based on various previous studies [38–40], the molecular size, shape, flexibility, polarity and charge of a compound seem to be most important for permeation through porins. Certain hydrogels, being highly versatile in their properties and adjustable in their gel network density, would be suitable materials to mimic this process. The importance of natural hydrogels such as mucus and bacterial biofilms to limit the access of antibiotics to their targets has already been pointed out by the work of Ribbeck et al.[41,42]. Moreover, polysaccharide hydrogels are also essential components for a wide range of analytical methods and applications, such as size exclusion chromatography and gel electrophoresis. This led us to the hypothesis that polysaccharide gels may feature fundamental separation criteria relevant also for permeability assessments of antibiotics into Gram-negative bacteria.

Polysaccharides being regularly employed for analytical and preparative methods based on size exclusion have several advantages, because of their gel-forming properties, low material costs and their physicochemical as well as structural diversity. This allows for imitation and investigation of various additional factors involved in passive compound permeation. Based on these considerations, we selected the four polysaccharides alginate, chitosan, slightly acid-degraded potato starch and agarose as gel-forming agents. We coated 96-well filters with these hydrogels using different concentrations and initially investigated the permeation of four representative antibiotics through those coatings. Out of these substances, ciprofloxacin (CIP) and tetracycline (TET) are reportedly high-permeating compounds in the Gram-negative model bacterium *Escherichia coli*, whereas rifampicin (RIF) and novobiocin (NOV) belong to low-accumulating compounds [40]. Collecting time-resolved permeation data allowed for calculating apparent permeability coefficients (P_{app}) across the different coatings. This parameter represents the speed of a molecule during its passage through an interface and commonly covers a wide range of measured permeation data, which leads to a more robust endpoint. Without any coating, permeation across the plain filter is rather fast and seems to favour reportedly low-accumulating NOV and RIF (Figure S1). In contrast, permeation of the same compounds across the different gel coatings tested was largely in agreement with their known bacterial uptake. As we found, however, the 20% (w/v) starch gel performed best (Figure S2 A) in distinguishing compounds by their permeability. Charge, as present in alginate and
chitosan (Figure S2 B, C), did not seem to play a crucial role. In the case of chitosan, the degree of deprotonation of the amine groups at neutral pH was obviously still too low to affect permeability. Freeze-fracture images suggest that in comparison to the starch network, structures might be too wide (e.g., chitosan, Figure S3 A–B) or too narrow (e.g., alginate, Figure S3 C) to make a difference. Agarose as a further uncharged polymer, forms a regularly meshed hydrogel network with larger pores (>100 nm, Figure S3 D), obviously too large to discriminate the permeability of small antibiotic molecules. Additional permeation studies on the two starch components amylose and amylopectin (Figure S2 E, F) demonstrate that discrimination between CIP and TET on the one hand and NOV and RIF on the other hand is more pronounced by the branched polysaccharide amylopectin, but still not as effective as a blend of both. The latter, according to our findings, leads to slightly denser polysaccharide networks of mixed and homogeneously distributed pore sizes (compare Figure 2 B, E to Figure S3 E, F). Given that the number of polysaccharide meshes smaller than 100 nm is visibly higher within the 20% starch gel, it is likely that smaller molecules such as ciprofloxacin with a length of around 10 nm cannot build up substantial dipole-dipole interactions. Molecules such as novobiocin with a diameter of around 20 nm, however, are in closer proximity to the surrounding hydroxyl moieties of the glucose units to build up stronger dipole-dipole interactions leading to the retention of the molecule. This may explain the good discriminating performance of 20% (w/v) starch gels. Lower starch concentrations as well as higher concentrations may again lead to suboptimal network densities (Figure 2 A, D and C, F).
Figure 2. TEM of replicas obtained from freeze-fractured, etched and replicated vitrified starch gel samples. Whereas 10% (w/v) retrograded starch gels show a comparatively wide-meshed secondary structure (A, D), 20% (w/v) starch gels contain a denser network of amylose and amylopectin double helices (B, E) leading to a more accurate differentiation between antibiotic permeabilities. Further increase of concentration (C, F) causes an even denser network but no improvement in differentiation. Pictures A-C: 68,000x magn., D-F: 98,000x magn. Colors were inverted. Scale bars represent 100 nm.

The additional investigation of the high-accumulating chloramphenicol (CHL) and the low-accumulating clindamycin (CLI) on the 20% starch formulation also leads to an accurate separation by their $P_{\text{app}}$ (Figure 3). This is noteworthy considering that CLI and TET are of similar molecular mass (444.44 Da vs. 424.98 Da, respectively). Moreover, it indicates that additional factors other than solely molecular size must be considered to explain their different permeability coefficients.
Figure 3. Permeation through a 20% starch membrane (A) in comparison to reported accumulation in *E. coli* MG1655 (B). Permeation coefficients of high accumulating CIP, TET and CHL as well as low accumulating RIF, NOV and CLI are in qualitative agreement with *in silico* compound accumulation. Columns in (A) represent mean \( P_{\text{app}} \pm \text{SEM} \), \( n = 9\)–11 from 3–4 independent experiments; a one-way ANOVA was performed with Tukey’s post-hoc analysis. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Columns in (B) are mean accumulated amounts \( \pm \text{SEM} \), as reported. [40]

3.2 Printing of polysaccharide gel on filter membranes

Before investigating a larger set of antibiotics with such set up, the membrane preparation was automated using a customized modular and multifunctional printer (Figure S4). This printer features a heated Hamilton® syringe as a print head, allowing to extrude and distribute equal amounts of 40 \( \mu \text{L} \) starch solution into each well before gel formation.

Topological characteristics of the starch coatings were investigated by scanning their absorbance in a plate reader at 666 nm after staining with methylene blue. For each well, 177 different locations were measured in a filled circle pattern. In our studies, printed membranes generally had a higher average absorbance per well than manually pipetted membranes (Figure 4 A, B; Figure S5 A) indicating an increased thickness. Moreover, the starch distribution of printed coatings was more homogenous, both within and between the produced batches (Figure S5 B-D; Figure S6 A, B). In addition, the formation of a meniscus was less prominent after printing (Figure 4 A, B). In order to evaluate the accuracy of the printed membrane model, we studied the permeation of the previously employed panel of CIP, TET, RIF and NOV on printed starch
membranes and compared the results to those manually prepared (Figure 4 C, S7).
While the permeability pattern of the four selected antibiotics was essentially the same, permeation of tetracycline through the printed starch membrane was decreased, reflecting that printed coatings were slightly thicker than pipetted ones. The standard deviation of the obtained permeability coefficients did not noticeably change, when the starch gel was printed, suggesting that the main causes of errors do not happen during the coating process.
Figure 4. Characterization and comparison of printed and pipetted membranes.

Three-dimensional absorbance intensity plot of a representative pipetted (A) and printed (B) starch membrane. A higher and more homogenous absorbance distribution of the starch membrane account for an enhanced layer thickness and a less intense meniscus formation. Structural differences had minor impact on the permeation of CIP, TET, RIF and NOV (C). Columns represent mean $P_{\text{app}} \pm \text{SD}$. A two-way ANOVA was performed with Tukey’s post-hoc analysis. $n = 12$ from 3 independent experiments.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001
3.3 Validation of the assay

After automation of the membrane-coating process, we selected 27 antibiotics and generated *in vitro* membrane-permeability data to validate the model (Figure 5 A, Table S4). Among those, the *in vitro* permeability of 16 compounds was compared to their *in bacterio* accumulation in *E. coli* K-12 strain MG1655 using the 10 minutes time point, obtained not only from literature [40], but also from own experiments (Figure 5 B).
Figure 5. Antibiotic permeation *in vitro* compared to *in bacterio* accumulation. A) Permeation-time course of 27 antibiotics across the starch based *in vitro* model, showing their different permeation kinetics (Error bars not shown; full dataset in Table S4). B) Quadrant plot of obtained permeated amounts from the *in vitro* starch model and their accumulation *in bacterio*. The *in bacterio* as well as the *in vitro* data show a separation into high (green quadrant) and low accumulating (red quadrant) antibiotics. To correct for a four times higher initial drug concentration used for the self-generated data (red circles), the accumulated amounts were multiplied by 0.25. Blue squares depict reported *in bacterio* accumulation [40]. Points represent mean permeated amounts ± SEM, $n_{in\;vivo}$ = 11–16 from 3–4 independent experiments. $n_{in\;bacterio}$ = 4 from 2 biological replicates.
Notably, all compounds could be consistently divided into a high-accumulating (green area) and a low-accumulating group (red area). TET, CIP, CLI and NOV served to demonstrate the comparability of own and reported \textit{in bacterio} data. A clear separation between high-accumulating CIP and TET and low-accumulating NOV and CLI, respectively, is noticeable and confirms a comparable outcome. Both for literature as well as own data, the \textit{in bacterio} accumulation is obviously in agreement with the permeated amounts according to the starch-based \textit{in vitro} assay after the same amount of time.

By looking more specifically at the gyrase inhibitors nalidixic acid (NAL) and CIP, both reach a high permeability \textit{in vitro} as well as a high level of accumulation \textit{in bacterio}, even though NAL accumulates more slowly, as can also be seen in the accumulation time course (Figure S8). Previous permeation studies on a multi-layered lipid \textit{in vitro} model already indicated a relatively fast permeation of NAL across the cell envelope of \textit{E. coli} [20]. Obviously, passive porin-independent permeation plays a significant role for this compound. Compared to TET, tigecycline (TIG) shows a slow but steadily increasing accumulation and permeation. Its 9-\textit{t}-(butylglycylamido) moiety, seems to sterically delimit the access via the unspecific bacterial porins OmpC and OmpF [43] and also impede the permeation through the starch network \textit{in vitro}. Additionally, TIG is known for its decreased efflux, mediated by its side chain [44], which explains its steadily increasing accumulated amount over time without fluctuation \textit{in bacterio}.

Another class that turned out to be low accumulating is the aminoglycosides. In agreement to their low accumulation, low permeation of tobramycin (TOB) and streptomycin (STR) can be observed in our \textit{in vitro} model, which is probably due to hydrogen bond formation with the hydroxyl-groups of the glucose units of starch. The same effect may occur with LPS, while aminoglycosides permeate across the outer membrane in course of ‘self-promoted uptake’ [11]. Clindamycin and lincomycin, which both feature a carbohydrate structure, may suffer from the same mechanism of retention like aminoglycosides. Notably, we observed low accumulation of sulfamethoxazole (SUL) in \textit{E. coli} despite its low molecular size. Since its entry route is so far largely unknown, one can only speculate that it is perhaps the absence of a positive charge or a zwitterionic structure, that delimits its permeation across OmpC and OmpF. Comparing the accumulation-time course \textit{in bacterio} to the permeation-time course \textit{in vitro} reveals the highest accuracy of the \textit{in vitro} model within the early time interval of 10 – 30 min (Figure 5 A, S8). Obviously, in absence of any active-
transport mechanisms, this assay may, however, not provide valid data for antibiotics that undergo TonB-dependent transport, such as cefiderocol and other future siderophore antibiotics. Peptides with membrane-disruptive or pore-forming effects, such as colistin, polymyxin B and daptomycin, are unsuitable test components for this model since their mechanism of action is strongly dependent on the presence of membrane phospholipids. As their target is the bacterial membrane itself, the investigation of their translocation is not required.

3.4 Structure–permeability relationships

Since a non-linear phase of permeation behaviour occurred for some compounds, the area under the curve (AUC_{10-30\text{ min}}) was calculated (Dataset S1) and used instead of P_{app} for investigations regarding structure–permeability relationships. Besides, a threshold value was introduced classifying substances with an AUC_{10-30\text{ min}} < 21 nmol*min as low permeating, whereas all other molecules were regarded as high-accumulating (Table S5). This threshold leads to the most accurate classification of all previously discussed compounds.

On this basis, we investigated structure–permeability relationships from our in vitro model and compared the outcome to currently discussed factors that determine compound accumulation in Gram-negative bacteria [6,36,45–47].

In line with previous findings in bacterio [38,45], molecular mass (M_w) also determines the permeability of compounds through our gel model. A decrease in permeation can be observed, when plotting the molecular mass of our panel of antibiotics against their AUC_{10–30\text{ min}} (Figure 6 A) with an apparent cut-off of at circa 500 Da. This value seems lower than the widely assumed permeation cut-off of ca 600 Da [8,48,49]. Accumulation, however, is – apart from uptake – also a function of efflux. Studies by Brown et al. indicate that especially those compounds with M_w between 450 Da and 600 Da undergo efflux in E. coli and Pseudomonas aeruginosa [46]. This condition might have contributed to the good match between in vitro permeation and in bacterio accumulation. Another distinct dependency was found between minimum projection area and drug permeation (Figure 6 B). This value being a hybrid parameter for molecular mass and three-dimensionality may be particularly helpful in drug
development, since it implies that a potentially low-accumulation due to high molecular mass can be compensated by reducing the spatial molecular expansion.
Figure 6. Correlations between drug permeation and selected physicochemical parameters. From 27 antibiotics, the AUC_{10-30 min} as representative parameter for permeation is plotted against (A) molecular mass, (B) minimum projection area, (C) globularity, (D) number of hydrogen bond donors and acceptors, (F) net charge, (G) type of amine group, (H) number of rotatable bonds, (I) relative abundance of unsaturated bonds, (J) \textit{clogD}_{pH7.4}. The dashed line represents the set AUC_{10-30min} threshold of 21 nmol*min.
Similar conclusions were drawn already recently [47]. By looking at the dependency between permeation and globularity a correlation can also be observed, even though it is less strong, if compared to the previous parameters (Figure 6 C).

As for rigidity, unlike previously reported [40], no clear correlation could be found between AUC_{10–30 min} and number of rotatable bonds (Figure 6 D). However, it seems plausible to use rather a relative parameter that is normalized to differences in molecular size. By considering the relative number of unsaturated bonds instead, we could indeed demonstrate a correlation between molecular rigidity and AUC_{10–30 min} (Figure 6 E), indicating that more rigid molecules permeate better. In contrast, no clear tendency was found among compounds with amine groups: only two compounds with a primary amine showed high accumulation, whereas nine of the high-accumulating drugs did not feature any primary amine. Moreover, seven compounds with a general amine (primary, secondary, or tertiary) highly accumulated, whereas twelve compounds featuring an amine accumulated low (Figure 6 F).

Rather impressively for a non-charged membrane, molecular net charge appears to affect permeation. A permeation optimum was reached at a net charge close to zero (Figure 6 G). This phenomenon may be attributed to ion–dipole interactions of permeating molecules to the hydroxyl groups of the polysaccharide network. In this regard, it is important to mention that most of the well-permeating drugs in vitro are zwitterions at pH 7.4 (8 out of 11), whereas the majority of low-accumulating antibiotics is not (3 out of 14). The preferred permeation of zwitterionic compounds was also reported for the porin OmpF [39], where, however, ion-ion interactions determine the translocation [17].

In line with earlier studies [45], we observed that the number of hydrogen bond donors and acceptors had impact on antibiotic permeation in vitro (Figure 6 H, I). In both cases, a low number was associated to better permeation. Notably, clogD_{pH7.4} as parameter for hydrophilicity or lipophilicity, also seemed to influence drug permeation, since molecules with clogD_{pH7.4} values between 0 and −5 permeated best (Figure 6 J). This is in agreement with previous assumptions about an enhanced permeation of slightly hydrophilic compounds as a typical feature of porin-mediated uptake [6,23,45].

After finding evidence for the impact of the aforementioned physicochemical properties, we employed a random-forest (RF) regression model to objectively confirm and rank these properties according to their impact on in vitro permeability. Our RF was generally trained with 26 out of 27 tested compounds, whereas the AUC_{10–30 min} of
the 27\textsuperscript{th} compound was predicted to be high- or low-accumulating. This was done in 27 cycles, each time predicting the AUC\textsubscript{10-30 min} of another 27\textsuperscript{th} compound after training the model with the remaining 26 substances. To assess the impact of the ten physicochemical parameters on the prediction, we systematically left one parameter out of the RF and compared the increase of prediction error (%IncMSE). In this way, the first RF regression confirmed seven factors (molecular mass, minimum projection area, rigidity, number of hydrogen bond donors and acceptors, globularity, charge) to be critical, for which the process was repeated. With this run, we obtained a ranking of the parameters according to their importance for the prediction (Table 1).

Table 1. Most influential physicochemical parameters according to random forest analysis.

| Feature                              | %IncMSE |
|--------------------------------------|---------|
| Minimum projection area              | 40.76   |
| Molecular mass                       | 27.99   |
| Relative abundance of unsaturated bonds | 16.23  |
| Number of hydrogen bond acceptors   | 5.80    |
| Net charge                           | 5.19    |
| Number of hydrogen bond donors      | 3.26    |
| Globularity                          | 0.71    |

Parameters representing molecular size and rigidity had the highest impact. Interestingly, \textit{clogD\textsubscript{pH7.4}} did not appear as an important parameter. This might be due to the rather small number of antibiotics used to train the RF model. Nonetheless, we found the results of our RF model in large agreement with our \textit{in vitro} and \textit{in bacterio} results.

When applying the previously mentioned threshold of 21 nmol\textsuperscript{\textast}min on the predicted AUC\textsubscript{10-30 min}, we achieved an accuracy of 88.9\% compared to AUC\textsubscript{10-30 min} obtained by our \textit{in vitro} assay and 81.3\% compared to \textit{in bacterio} compound accumulation (Table S5).
3.5 Application

Having demonstrated associations between in vitro permeation and in bacterio accumulation, we applied our assay to in-house small-molecule RNA polymerase inhibitors 1–3 with potent activity against Gram-positive bacteria [32] but limited activity against Gram-negative species (Table 2). By inspecting the ratio of target inhibition (IC_{50}) and antibacterial activity (MIC_{95}) against the efflux mutant E. coli ΔTolC, it is obvious that compounds 1 and 2 can access the intracellular target better than 3 (IC_{50}/MIC_{95} ratio: 0.42, 0.96, and < 0.16, respectively). Indeed, this difference corresponded to the permeability data of our in vitro model (Figure 7).

Table 2. RNA polymerase inhibitors and their antibacterial and physicochemical properties.

| ID | Structure | Activity | Permeability | Molecular Descriptors |
|----|-----------|----------|--------------|-----------------------|
|    |           | IC_{50}  | MIC_{95}      | P_{app} *10^{-5} | Min. proj. area [Å²] | Mw [Da] | Rigi-dity | # HBA | Net charge (pH 7.4) | # HBD | Glob. |
| 1  | ![Structure](image1) | 14       | 33           | 0.42             | 0.37 ± 0.03           | 39.07   | 425.3    | 0.43 | 6               | -1   | 3     | 0.011 |
| 2  | ![Structure](image2) | 22       | 23           | 0.96             | 0.34 ± 0.06           | 51.12   | 437.3    | 0.45 | 5               | -1   | 3     | 0.013 |
| 3  | ![Structure](image3) | 8        | >50          | <0.16            | 0.20 ± 0.08           | 60.86   | 562.4    | 0.48 | 8               | -1   | 4     | 0.050 |

*half inhibitory concentration of E. coli RNA polymerase; MIC values against E. coli tolC (efflux mutant).
Figure 7. Assessment of the permeability of RNA polymerase inhibitors 1–3. The comparison to high accumulating CIP and low accumulating NOV indicates that all presented RNAP-inhibitors likely belong to low accumulating drugs, whereas 3 probably accumulates particularly low. Columns represent mean $P_{\text{app}} \pm \text{SEM}$; $n = 9–12$ from 3 independent experiments. One-way ANOVA was performed with Tukey’s post-hoc analysis. ****P<0.0001, **P<0.01.

Notably, the permeability of all three compounds was rather low although their molecular mass is lower than novobiocin (613 Da). This is plausible considering the absence of a positive charge, which would be necessary for the formation of a zwitterion and neutralization of the net charge. Moreover, the lipophilicity of these compounds is rather high ($\text{clogD}_{\text{pH}7.4} > 2$). The particularly slow permeation of compound 3 is understandable, since it is the largest, most lipophilic and most globular compound of all three RNA polymerase inhibitors. The overall low permeability of these compounds is in agreement with their MIC$_{95}$ values and explains the weak activity of this class against Gram-negative bacteria. While all our readouts were obtained by directly measuring absorbance in a plate reader, the model may also be expanded to contemporary LC–MS methods [50].
3.6 Predicting activity in different bacterial strains

Finally, we wanted to explore direct associations between \textit{in vitro} permeability and antibacterial activity against Gram-negative bacterial species mentioned in the priority list by the World Health Organization [51]. For this purpose, we compared reported maximum MIC values from the European Committee for Antimicrobial Susceptibility Testing (EUCAST) data base for \textit{E. coli}, \textit{P. aeruginosa}, \textit{Acinetobacter baumannii}, \textit{Klebsiella pneumoniae}, \textit{Campylobacter jejuni}, \textit{Salmonella spp.} and \textit{Neisseria gonorrhoeae} [52] with the $P_{\text{app}}$-values for 27 compounds as measured by the starch hydrogel assay (Figure 8, Dataset S1).
Figure 8. Quadrant plot of *in vitro* permeability and *in bacterio* activity relationships for *Escherichia coli* (A), *Acinetobacter baumannii* (B), and *Klebsiella pneumoniae* (C), and *Pseudomonas aeruginosa* (D), *Campylobacter jejuni* (E), *Salmonella spp.* (F) and *Neisseria gonorrhoeae* (G). Arbitrarily set lines at an apparent permeability coefficient ($P_{app}$) of $1 \times 10^{-5}$ cm/s and a minimum inhibitory concentration (MIC) of 64 µg/mL demonstrate that, for the majority of all 27 candidate antibiotics tested, low MIC values are associated with high permeability (green quadrant). *Vice versa*, low *in vitro* permeability is associated with poor antibacterial activity. Only few points show activity in spite of low *in vitro* permeability (red quadrant = false negatives). Crosses indicate non-available MICs according to EUCAST standards and were arbitrarily set to >256 µg/mL.
Obviously, throughout all seven species, antibacterial activity of a given compound is - with very few exceptions - associated with a $P_{\text{app}}$ above $10^{-5}\text{ cm/s}$. For antibiotics with intracellular targets, permeability across the Gram-negative bacterial cell envelope represents a necessary but not sufficient condition for antibacterial activity. Measuring the $P_{\text{app}}$-value \textit{in vitro} allows identifying compounds, which are unlikely to be active \textit{in bacterio}. Compounds with antibiotic activity despite low permeability, would therefore represent false negatives of such assay. However, those were only two to three of 27 compounds in \textit{E. coli}, \textit{A. baumannii}, \textit{P. aeruginosa} and \textit{K. pneumoniae}. This simple \textit{in vitro} assay therefore opens the perspective not only to predict \textit{in bacterio} accumulation, but also to exclude probably inactive antibiotics at an early stage of drug development.

\section*{Conclusion}

We investigated different polysaccharide hydrogels with the aim to find functional properties that allow for the prediction of antibiotic accumulation in Gram-negative bacteria. Freeze-fracture TEM of gel-networks from different polysaccharides and concentrations revealed a remarkable diversity of network structures and porosity. A 20\% (w/v) starch hydrogel model proved competent to discriminate high- from low-accumulating antibiotics in the Gram-negative model bacterium \textit{E. coli}.

The preparation of the model, regardless if manually pipetted or printed, is simple, reproducible, cost-effective, and hazard-free. While uptake studies in living bacteria or artificial vesicles are cumbersome to perform, membrane-permeation experiments can be automated and deliver accurate results within 10 minutes, making it compatible with high-throughput screening applications on molecules with various physicochemical properties.

Applying contemporary tools of machine learning to our \textit{in vitro} data provided evidence to the impact of molecular characteristics, which were reported earlier \textit{in bacterio}. Although due to obvious differences between the chosen polysaccharide and the bacterial cell envelope, we found that a small set of seven features was sufficient of create a robust machine-learning model with good prediction based on \textit{in vitro} permeation data. In the context of studying \textit{in vitro} permeability of structurally diverse antibiotics, we also report the first data on \textit{in bacterio} accumulation of aminoglycosides and sulphonamides – important classes of antibiotics to treat Gram-negative infections.

By applying the gel-based assay on in-house synthesized antibiotics, we could find an
explanation for their low activity against Gram-negative bacteria. Expanding the investigations to antibiotic activity against highly relevant Gram-negative species gave evidence that *in vitro* permeability data may allow to exclude inactive substances at an early stage of antibiotic development.

Room for further improvements of the model may consist in the use of starches with different ratios of amylose and amylopectin as well as further variation of gel concentrations and charges to obtain a more pathogen-specific prediction. The addition of β-lactamases or penicillin binding proteins to the acceptor compartment may lead to enhanced accuracy regarding β-lactam antibiotics. Prospectively, by refining the composition of the alginate formulation or the application of biological hydrogels, the described assay can be modified towards investigating permeability across biofilms, exopolysaccharides or mucus on larger scale.

5. Acknowledgments

We would like to thank Pascal Paul and Sarah Pawusch (Helmoltz Institute for Pharmaceutical Research Saarland, HIPS) for support with LC–MS analysis and support with the analysis of *in vitro* permeability-activity relationships, Henni-Karoliina Ropponen (HIPS) and Carla F. Sousa (HIPS) for calculating physicochemical parameters, Dr. Sven-Kevin Hotop (Helmholtz Centre for Infection Research, HZI) for support with LC–MS and additional measurements on tobramycin, Jennifer Herrmann and Prof. Rolf Müller (both HIPS) for providing amidochelocardin and sorangicin A, Prof. Gerhard Wenz (Department of Organic Macromolecular Chemistry, Saarland University) for providing slightly acid-degraded starch and AVEBE (Veendam, NE) for kindly donating amylopectin. This work was supported by the Helmholtz Research Program “Infection Research”.

6. References

[1] C. Nathan, O. Cars, Antibiotic resistance - Problems, progress, and prospects, N. Engl. J. Med. 371 (2014) 1761–1763. https://doi.org/10.1056/NEJMp1408040.

[2] L.L. Silver, Challenges of Antibacterial Discovery, Clin. Microbiol. Rev. 24 (2011) 71–109. https://doi.org/10.1128/CMR.00030-10.

[3] B. Aslam, W. Wang, M.I. Arshad, M. Khurshid, S. Muzammil, M.H. Rasool, M.A. Nisar, R.F. Alvi, M.A. Aslam, M.U. Qamar, M.K.F. Salamat, Z. Baloch,
H. Bajaj, S. Acosta Gutierrez, I. Bodrenko, G. Malloci, M.A. Scorciapino, M. Winterhalter, M. Ceccarelli, Bacterial Outer Membrane Porins as Electrostatic Nanosieves: Exploring Transport Rules of Small Polar Molecules, ACS Nano. 11 (2017) 5465–5473. https://doi.org/10.1021/acsnano.6b08613.

J. Wang, R. Terrasse, J.A. Bafna, L. Benier, M. Winterhalter, Electrophysiological characterization of transport across outer membrane channels from Gram-negative bacteria in presence of lipopolysaccharides (LPS), Angew. Chemie Int. Ed. (2020) 1–6. https://doi.org/10.1002/anie.201913618.

F. Graef, B. Vukosavljovic, J.P. Michel, M. Wirth, O. Ries, C. De Rossi, M. Windbergs, V. Rosilio, C. Ducho, S. Gordon, C.M. Lehr, The bacterial cell envelope as delimiter of anti-infective bioavailability – An in vitro permeation model of the Gram-negative bacterial inner membrane, J. Control. Release. 243 (2016) 214–224. https://doi.org/10.1016/j.jconrel.2016.10.018.

F. Graef, R. Richter, V. Fetz, X. Murgia, C. De Rossi, N. Schneider-Daum, G. Allegretta, W. Elgaher, J. Haupenthal, M. Empting, F. Beckmann, M. Brönstrup, R. Hartmann, S. Gordon, C.M. Lehr, In Vitro Model of the Gram-Negative Bacterial Cell Envelope for Investigation of Anti-Infective Permeation Kinetics, ACS Infect. Dis. 4 (2018) 1188–1196. https://doi.org/10.1021/acsinfecdis.7b00165.

T. Nakae, Outer Membrane of Salmonella Typhimurium: Reconstitution Of Sucrose-Permeable Membrane Vesicles, Biochem. Biophys. Res. Commun. 64 (1975) 1224–1230.

O. Ries, C. Carnarius, C. Steinem, C. Ducho, Membrane-interacting properties of the functionalised fatty acid moiety of muraymycin antibiotics, Medchemcomm. 6 (2015) 879–886. https://doi.org/10.1039/c4md00526k.

W. Zimmermann, A. Rosselet, Function of the outer membrane of Escherichia coli as a permeability barrier to beta lactam antibiotics, Antimicrob. Agents Chemother. 12 (1977) 368–372. https://doi.org/10.1128/AAC.12.3.368.

G. Decad, T. Nakae, H. Nikaido, Permeability of Escherichia coli and Salmonella typhimurium cell wall to Oligosaccharides, Fed Proc. 33 (1974) 1240.

H. Prochnow, V. Fetz, S.K. Hotop, M.A. García-Rivera, A. Heumann, M. Brönstrup, Subcellular Quantification of Uptake in Gram-Negative Bacteria, Anal. Chem. 91 (2019) 1863–1872. https://doi.org/10.1021/acs.analchem.8b03586.

R. Iyer, M.A. Sylvester, C. Velez-Vega, R. Tommasi, T.F. Durand-Reville, A.A. Miller, Whole-Cell-Based Assay to Evaluate Structure Permeation Relationships for Carbapenem Passage through the Pseudomonas aeruginosa Porin OprD, ACS Infect. Dis. 3 (2017) 310–319. https://doi.org/10.1021/acsinfecdis.6b00197.

J.D. Prajapati, C.J. Fernández Solano, M. Winterhalter, U. Kleinekathöfer, Characterization of Ciprofloxacin Permeation Pathways across the Porin OmpC Using Metadynamics and a String Method, J. Chem. Theory Comput. 13 (2017) 4553–4566. https://doi.org/10.1021/acs.jctc.7b00467.
[28] S. Acosta-Gutiérrez, L. Ferrara, M. Pathania, M. Masi, J. Wang, I. Bodrenko, M. Zahn, M. Winterhalter, R.A. Stavenger, J.M. Pagès, J.H. Naismith, B. Van Den Berg, M.G.P. Page, M. Ceccarelli, Getting Drugs into Gram-Negative Bacteria: Rational Rules for Permeation through General Porins, ACS Infect. Dis. 4 (2018) 1487–1498. https://doi.org/10.1021/acsinfecdis.8b00108.

[29] K.R. Pothula, C.J.F. Solano, U. Kleinekathöfer, Simulations of outer membrane channels and their permeability, Biochim. Biophys. Acta - Biomembr. 1858 (2016) 1760–1771. https://doi.org/10.1016/j.bbamem.2015.12.020.

[30] C.F. Sousa, J.T.S. Coimbra, I. Gomes, R. Franco, P.A. Fernandes, P. Gameiro, The binding of free and copper-complexed fluoroquinolones to OmpF porins: an experimental and molecular docking study, RSC Adv. 7 (2017) 10009–10019. https://doi.org/10.1039/C6RA26466B.

[31] R. Tommasi, R. Iyer, A.A. Miller, Antibacterial Drug Discovery: Some Assembly Required, ACS Infect. Dis. 4 (2018) 686–695. https://doi.org/10.1021/acsinfecdis.8b00027.

[32] W.A.M. Elgaher, K.K. Sharma, J. Haupenthal, F. Saladini, M. Pires, E. Real, Y. Mély, R.W. Hartmann, Discovery and Structure-Based Optimization of 2-Ureidothiophene-3-carboxylic Acids as Dual Bacterial RNA Polymerase and Viral Reverse Transcriptase Inhibitors, J. Med. Chem. 59 (2016) 7212–7222. https://doi.org/10.1021/acs.jmedchem.6b00730.

[33] A. Liaw, M. Wiener, Classification and Regression by randomForest, R News. 2 (2002) 18–22. https://doi.org/10.1002/sres.1082.

[34] W. Humphrey, A. Dalke, K. Schulten, Sartorius products, J. Mol. Graph. 14 (1996) 33–38. https://doi.org/10.1016/0263-7855(96)00018-5.

[35] T.J. Silhavy, D. Kahne, S. Walker, The bacterial cell envelope, Cold Spring Harb. Perspect. Biol. 2 (2010) : a000414. https://doi.org/10.1101/cshperspect.a000414.

[36] M.F. Richter, P.J. Hergenrother, The challenge of converting gram-positive-only compounds into broad-spectrum antibiotics, Ann. N. Y. Acad. Sci. 1435 (2019) 18–38. https://doi.org/10.1111/nyas.13598.

[37] S. Acosta-Gutiérrez, I. Bodrenko, M. Ceccarelli, Permeability through bacterial porins dictates whole cell compound accumulation., ChemRxiv. (2020). https://doi.org/https://doi.org/10.26434/chemrxiv.11877834.v1 Preprint.

[38] G.M. Decad, H. Nikaido, Outer membrane of gram negative bacteria. XII. Molecular sieving function of cell wall, J. Bacteriol. 128 (1976) 325–336.

[39] F. Yoshimura, H. Nikaido, Diffusion of β-lactam antibiotics through the porin channels of Escherichia coli K-12, Antimicrob. Agents Chemother. 27 (1985) 84–92. https://doi.org/10.1128/AAC.27.1.84.

[40] M.F. Richter, B.S. Drown, A.P. Riley, A. Garcia, T. Shirai, R.L. Svec, P.J. Hergenrother, Predictive compound accumulation rules yield a broad-spectrum antibiotic, Nature. 545 (2017) 299–304. https://doi.org/10.1038/nature22308.

[41] O. Lieleg, K. Ribbeck, Biological Hydrogels as Selective Diffusion Barriers, Trends Cell Biol. 21 (2011) 543–551. https://doi.org/10.1016/j.tcb.2011.06.002.
K.M. Wheeler, G. Cárcamo-Oyarce, B.S. Turner, S. Dellos-Nolan, J.Y. Co, S. Lehoux, R.D. Cummings, D.J. Wozniak, K. Ribbeck, Mucin glycans attenuate the virulence of Pseudomonas aeruginosa in infection, Nat. Microbiol. 4 (2019) 2146–2154. https://doi.org/10.1038/s41564-019-0581-8.

I. Chopra, M. Roberts, Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance, Microbiol. Mol. Biol. Rev. 65 (2001) 232–260. https://doi.org/10.1128/MMBR.65.2.232.

Y. Someya, A. Yamaguchi, T. Sawai, A novel glycyclcline, 9-(N,N-dimethylglycylamido)-6-demethyl-6-deoxytetracycline, is neither transported nor recognized by the transposon Tn10-encoded metal-tetracycline/H+ antiporter, Antimicrob. Agents Chemother. 39 (1995) 247–249. https://doi.org/10.1128/aac.39.1.247.

R. O'Shea, H.E. Moser, Physicochemical properties of antibacterial compounds: Implications for drug discovery, J. Med. Chem. 51 (2008) 2871–2878. https://doi.org/10.1021/jm700967e.

D.G. Brown, T.L. May-Dracka, M.M. Gagnon, R. Tommasi, Trends and exceptions of physical properties on antibacterial activity for gram-positive and gram-negative pathogens, J. Med. Chem. 57 (2014) 10144–10161. https://doi.org/10.1021/jm501552x.

F. Ruggiu, S. Yang, R.L. Simmons, A. Casarez, A.K. Jones, C. Li, J.M. Jansen, H.E. Moser, C.R. Dean, F. Reck, M. Lindvall, Size Matters and How You Measure It: A Gram-Negative Antibacterial Example Exceeding Typical Molecular Weight Limits, ACS Infect. Dis. 5 (2019) 1688–1692. https://doi.org/10.1021/acsinfecdis.9b00256.

H. Nikaido, Porins and specific diffusion channels in bacterial outer membranes, J. Biol. Chem. 269 (1994) 3905–3908.

H.I. Zgurskaya, C.A. López, S. Gnanakaran, Permeability Barrier of Gram-Negative Cell Envelopes and Approaches To Bypass It, Physiol. Behav. 1 (2015) 512–522. https://doi.org/10.1016/j.physbeh.2017.03.040.

M. Widya, W.D. Pasutti, M. Sachdeva, R.L. Simmons, P. Tamrakar, T. Krucker, D.A. Six, Development and Optimization of a Higher-Throughput Bacterial Compound Accumulation Assay, ACS Infect. Dis. 5 (2019) 394–405. https://doi.org/10.1021/acsinfecdis.8b00299.

World Health Organization, Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug resistant bacterial infections, including tuberculosis, WHO/EMP/IAU/2017.12. (2017).

European Committee on Antimicrobial Susceptibility Testing, EUCAST, https://mic.eucast.org/Eucast2, (2020). https://mic.eucast.org/Eucast2/ (accessed April 7, 2020).