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Quantitative characterization of binding of small molecules to extracellular matrix

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Abstract

Extracellular matrix (ECM) is a major tissue component that, besides its cell support function, is implicated in cell-cell signaling, wound repair, cell adhesion, and other cell and tissue functions. For small molecules acting in tissues, including chemicals, signaling peptides, effectors, inhibitors, and other man-made and physiological compounds, non-specific binding to ECM is a critical phenomenon affecting their disposition. We describe here a method for a quantitative characterization of the ECM binding, using a solidified ECM layer incubated with medium containing studied small molecules. Working conditions of Matrigel, a commercial basement membrane preparation were optimized in terms of the protein concentration, surface area, gel layer thickness, solidification time, and mixing speed. The release of proteins from the solidified layer into the buffer was monitored and taken into account. Two major proteins, laminin and collagen IV, dissolve at different rates. The Matrigel stability data, obtained under varying incubation conditions and gentle mixing, can also be useful in other ECM-related research. The experimental binding data, averaged over all binding sites, were analyzed assuming a fast linear binding. The binding constants were determined for ten small organic molecules for both dissolved proteins and the solidified layer. The binding constants tend to increase with lipophilicity of the compounds, as characterized by the 1-octanol/water partition coefficients.

Keywords

extracellular matrix; Matrigel; Matrigel dissolution; binding assay; lipophilicity; 1-octanol/water partition coefficient

1. Introduction

Basement membrane and connective tissues are two structural types of extracellular matrix (ECM). While connective tissue matrix features porous, fibrillar architecture, the basement membrane is a dense, sheet-like structure at the interface between epithelia and mesenchymal tissues, corresponding to its roles as a structural support and a selective barrier separating cells and proteins. Compositionally a viscous pool with collagens I, II, and IV, laminin, proteoglycans, and growth factors, the basement membrane provides for dynamic environmental communication between cells, which is important for cell-cell signaling, wound repair, cell adhesion, and other cell and tissue functions [1]. Breakdown of basement membrane is a precondition for e.g., angiogenesis, migration, and invasion of tumor cells. Inhibition of the breakdown process in these cases could be of therapeutic significance [2,3].

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This study reports the development of a method for quantification of ECM binding of small molecules acting in tissues, including signaling peptides, effectors, inhibitors, and other drug candidates. Throughout this paper, we will frequently refer to all these molecules as drugs. Reversible drug binding to non-target membranes and/or proteins in cellular or extracellular space creates a depot releasing the drug over a longer time period. Excessive binding affinity, however, can compromise drug activity by limiting bioavailability and the local concentration around receptors. While binding to membranes is frequently characterized and correlated to properties like lipophilicity and amphiphilicity [4-8], nonspecific binding to ECM and other proteins, except plasma albumin and α -acid-glycoprotein [9-12], has rarely been studied. Understanding of ECM binding is important for drug disposition in tissues.

Previous studies of ECM binding mostly concentrated on binding of macromolecules [13], peptides, or bacteria [14] to specific ECM components. Analytic methods include particle agglutination assay [14], affinity chromatography, immunosorbant assay [15], and isotope labeling [16].

Human ECM can be prepared from fetal dermal fibroblasts [17]. Commercially available Matrigel® is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in ECM proteins. Major components include laminin (56%) and collagen IV (31%), followed by heparan sulfate proteoglycans, entactin, nidogen, and several growth factors like transforming growth factor- β , fibroblast growth factor, tissue plasminogen activator, and other minor components [18]. Transmission electron microscopy revealed co-distribution of components with the appearance of 5-nm-wide cords [19]. Collagen IV forms the scaffold of a continuous network by forming intermolecular disulfide bonds [18]. Laminin binds to native, but not denatured collagen IV, by the short chain and through a domain in its long chain to the heparan sulfate proteoglycan [20]. Due to its ability to form a thin layer of gel quickly at 22-35°C and maintaining this form with culture media, according to the vendor, for a period of 12 days at 37°C, Matrigel has been used in lieu of the basement membrane in several assays, e.g. for tumor cell invasion [21-23], in vivo peripheral nerve regeneration [24], cell differentiation [25,26], and angiogenesis [27]. Matrigel has also been used in binding studies of recombinant fibronectin type II-like modules of human 72-kDa gelatinase/type IV collagenase [15].

In this study, we describe a simple method for a quantitative analysis of drug binding to Matrigel forming a thin gelled layer at the bottom of a test vial. The method accounts for dissolution of Matrigel components that also bind the studied compounds. The approach was used to analyze Matrigel binding for a series of simple aromatic compounds.

2. Material and methods

2.1. Materials and equipment

Matrigel, mouse laminin-1 and mouse collagen IV were all purchased from BD Biosciences, Labware Discovery (Bedford, MA); the D-MEM medium (phenol red free) was purchased from Invitrogen (Carlsbad, CA); primary antibodies for laminin-1 and collagen IV were purchased from Rockland (Gilbertsville, PA); the molecular weight standard was obtained from BioRad (Hercules, CA); simple aromatic compounds were purchased from Sigma (St. Louis, MO); Bradford reagent, buffer species, and all solvents were purchased from Sigma, analytical grade or spectroscopy grade.

All photometric and spectroscopy measurements were carried out on Shimadzu 1601 UV-Vis spectrophotometer. Elkay Ultra-Vu® Disposable Cuvettes from Elkay Products, Inc. (Shrewsbury, MA) were used in the Bradford assay [28]. Either open-top or septa sealed cuvettes from Nova Biotech (El Cajon, CA) were used in all other assays. The apparatus for

Western Blots was from BioRad. Band intensity of Western Blots was analyzed by FluoChemTM 5500 from Alpha Innotech (San Leandro, CA).

2.2. Preparation and use of solidified Matrigel layers

Original Matrigel preparation was diluted in the given ratio with the D-MEM medium using a pipette with icy tip. Two types of flat-bottom amber glass vials were used: 40-mL vials (O.D. 27 mm × 95 mm) and 2-mL vials (O.D. 12mm × 35mm). Diluted Matrigel (0.5 mL unless specified otherwise) was carefully deposited at the bottom of a set of vials on ice and incubated at 37°C for 2 hrs unless specified otherwise.

Matrigel preparation was initially used in the manufacturer-recommended manner (threefold dilution by the medium and a 30 minute solidification time) to prepare a 0.9 mm thick layer of gelled Matrigel at the bottom of 2-mL amber vials. When overlaid with borate buffer and incubated at 37 °C on a reciprocal shaker, a significant protein release and a severe deformation of the gelled layer were observed even at the lowest mixing speed (20 rpm) needed to ensure gradient-free distribution of tested compounds in the buffer. Among different Matrigel dilutions that were examined in a search for a more stable preparation, the Matrigel to medium ratio of 1:2.5, corresponding to protein concentration 5.76 mg/mL, showed the slowest protein release and was selected for all subsequent experiments, unless stated otherwise.

If the solution of a tested compound does not penetrate the Matrigel layer easily, the ratio of the Matrigel surface area to the buffer volume is an important factor for the extent of binding. The influence of this factor on relative binding was tested using two sizes of amber glass vials: 2-mL vials with dimensions of 12 (O.D.) × 35 mm and internal cross-section area of 0.77 cm², and 40-mL vials with dimensions 27 (O.D.) × 95 mm and internal cross-section area of 4.91 cm². Using approximately the same amount of Matrigel, the second setup exhibited more pronounced binding of tested compound and was used in all experiments.

The solidified Matrigel was overlaid with borate buffer (pH=7.4, 0.7 mL and 2 mL for 2-mL and 40-mL vials, respectively) with or without the studied compound and incubated at 37°C on a reciprocal shaker (Lab-Line Instruments Inc., Melrose Park, IL) for specified time with mixing speed 20 rpm unless specified otherwise. After incubation, a portion of the buffer was withdrawn and used for analysis of dissolved proteins (0.1 mL) or studied compounds (0.5 or 1 mL).

The Bradford assay [28] was used for protein content determination. For construction of the calibration line, Matrigel was diluted serially to 5 concentrations (114 - 570 µg/mL) with borate buffer (10 mM Na₂B₄O₇, 180 mM H₃BO₃, 18 mM NaCl, pH 7.4) [29]. Matrigel solution (0.1 mL) and Bradford reagent (3 mL) were mixed in a 3.5-mL disposable plastic cuvettes and let stand for 10 minutes. The calibration line was constructed using the absorbance measurement at 595 nm. The absorption coefficient, equal to the slope of the calibration line, was 4.349×10^{-4} mL/(µg×cm). For the analysis, Matrigel solution (0.1 mL) with unknown protein content was mixed with the Bradford reagent (3 mL) and processed as described above.

2.3. Kinetics of dissolution of solidified Matrigel layer

The solidified Matrigel layer was prepared in a set of vials and incubated with borate buffer as described above. After 0.5, 1, 2, 4, and 6 hour incubations, the supernatant containing dissolved proteins was withdrawn and the amounts of dissolved main Matrigel components, laminin and collagen IV, were evaluated by Western Blots using 20 µL samples. The sample were boiled with 10 µL of Laemmli [30] buffer (62.5 mM TRIS-HCL, pH 6.8, 2 % SDS, 25 % glycerol, 0.01 % Bromophenol Blue,) and 10µL of dithiothretol solution (0.1 M) for 10 minutes. SDS-PAGE was carried out by using a 7.5% acrylamide separating gel at 90 V. After

transferring to a polyvinylidene difluoride membrane at 100 V for two hours, primary antibody (1:4000 and 1:5000 dilution, or 25ng and 20ng, for laminin and collagen, respectively) was applied and left to bind at 4°C overnight. After careful rinsing, horseradish peroxidase labeled goat anti-rabbit IgG (1:5000 dilution, as suggested by vendor), was conjugated to the membrane for 45 minutes, with rocking. The membrane was taken out, exposed to Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences Inc., Boston, MA) for 1 minute with shaking, and imaged by FluoChem™ 5500 (Alpha Innotech, San Leandro, CA).

2.4. Determination of binding constants

For the determination of the absorption coefficients of compounds, the drug stock solution was prepared in DMSO and diluted 1:100 with borate buffer (pH 7.4). UV-Vis spectra were taken and absorption coefficients were calculated for wavelengths from 240 to 305 nm with 5-nm intervals. To determine the absorption coefficient, Matrigel was diluted to several concentrations with borate buffer containing 1% DMSO. Absorbances were plotted against concentration for each wavelength and the absorption coefficients were calculated as the slope. Throughout the paper, we use ϵ to denote the absorption coefficient multiplied by the light path, with the relevant species indicated in the subscript.

Evaporation of volatile chemicals was monitored as the decrease of drug concentration in borate buffer (1 % DMSO) in a sealed 3.5-mL cuvette or 40-mL glass vials as measured by UV-Vis absorbance. The Henry constant H was calculated using Eq. 1 in part 2.4.3.

2.4.1. Determination of association constant to diluted Matrigel (K_D)—Drug solution in borate buffer (2.4 mL, < 1 % DMSO) of suitable concentration was titrated with 20 μ L of diluted Matrigel (1152 μ g/mL), briefly vortexed and let stand for 5 minutes before the UV-Vis spectra measurement. The titration was repeated 6-12 times. For volatile compounds, the titration was carried out in a septum-capped standard quartz cuvette. The drug solution was adjusted to 4 mL for highly volatile benzene. Addition of Matrigel started after the evaporation equilibrium was established. Measurements at multiple wavelengths were used to obtain the absorption coefficient of drug-protein complex multiplied by the light path (ϵ_{DP}) and the association constant to diluted Matrigel proteins (K_D) by non-linear regression according to Eq. 5 (part 2.4.3). The initial and fitted values of ϵ_{DP} were expected to lie in the interval having the smaller of the values ϵ_P and ϵ_D as the lower limit and the sum $\epsilon_D + \epsilon_P$ as the upper limit.

2.4.2. Determination of association constants to dissolved (K_P) and solidified Matrigel (K_S)—Matrigel (0.5 mL, 5.76 mg/mL) was pipetted with ice-cooled pipette tips to the bottom of a 40-mL amber vial on ice and incubated at 37°C for 2 hours. Borate buffer (1.98 mL) was added and shaken with solidified Matrigel for 4 hours before addition of drug (20 μ L) in various concentrations (< 1 % DMSO). After shaking for another 2 hours, the top solution was taken to measure UV-Vis spectra. Samples of volatile compounds were taken in different times to determine when equilibria in the three-phase system were reached. The association constants to dissolved (K_P) and solidified Matrigel (K_S) were determined using the fit of Eq. 7 to experimental data.

2.4.3. Data processing—The drug molecules (D) dissolved in the buffer can bind to solidified Matrigel (subscript S) and to dissolved Matrigel proteins (P), as well as evaporate into the gas phase (subscript G). The corresponding equilibria in a closed system are depicted in Fig. 1.

The evaporation is characterized by the Henry constant $H = [D]_G/[D]$, with the square brackets denoting concentrations. Binding to dissolved Matrigel proteins is described by the association

constant $K_P = [DP]/[D] \times [P]$. Linear binding to solidified Matrigel is expressed by $K_S = [D]_S/[D]$, assuming that the concentration of binding sites in solidified Matrigel is much higher than total drug concentration so that the relative loss of binding sites upon binding is negligible.

Dimensionless Henry constant for volatile compounds---This quantity was determined directly from the definition:

$$H = \frac{[D_G]}{[D]} = \frac{(n_D - n_A) \times V_A}{n_A \times V_G} = \left(\frac{1}{c_A/c_D} - 1 \right) \times \frac{V_A}{V_G}. \quad (1)$$

Here, n are the drug amounts, c are the concentrations, and V s are the volumes. The subscripts A and G refer to the aqueous and gaseous phases, respectively. The subscript D indicates the total or initial drug amount or concentration in the aqueous phase. The ratio of actual and initial drug concentrations in the right-most part of Eq. 1 is easily measured as the ratio of actual and initial absorbances, providing that the volume of the aqueous phase does not significantly change upon evaporation.

Association constant for binding to diluted Matrigel (K_D)---The free drug concentration in the aqueous phase is equal to the total drug concentration c_D , reduced by evaporation and binding to diluted proteins that is characterized by the association constant K_D (Fig. 1):

$$[D] = c_D - H \times [D] \times V_G/V_A - K_D \times [D] \times c_P. \quad (2)$$

The evaporated drug concentration is expressed from the definition of the Henry constant H in the second term. The definition of the association constant K_D was used to express the protein bound concentration in the third term, assuming that the protein concentration $[P]$ is not significantly changing upon binding and can be expressed using the total protein concentration c_P . The free drug concentration in the aqueous phase can be separated from Eq. 2 as:

$$[D] = c_D / (1 + H \times V_G/V_A + K_D \times c_P) \quad (3)$$

The absorbance A of the mixture consists of contributions from free drug, diluted Matrigel protein, and from drug-protein complex. Each contribution can be written as the respective concentration multiplied by the product of the absorption coefficient and the light path (ϵ):

$$A = \epsilon_D \times [D] + \epsilon_{DP} \times [DP] + \epsilon_P \times c_P. \quad (4)$$

The free drug concentration is given by Eq. 3. The concentration of the drug-protein complex can be described using the free drug concentration (Eq. 3), multiplied by $K_D \times c_P$ as follows from the definition of the association constant K_D . Eq. 4 can then be rearranged as:

$$A = [(\epsilon_D + \epsilon_{DP} \times K_D \times c_P) \times c_D] / (1 + H \times V_G/V_A + K_D \times c_P) + \epsilon_P \times c_P. \quad (5)$$

Equation 5 was used to fit the total absorbance A as a function of total drug and protein concentrations (c_D and c_P) in the titrations with diluted Matrigel, in the absence of solidified Matrigel layer. The fitted parameters were ϵ_{DP} and K_D . The remaining quantities (H , ϵ_D , ϵ_P) were determined in independent experiments. For non-volatile compounds, $H = 0$.

The association constant K_S for the solidified Matrigel---The description is similar to that for binding to diluted proteins in the previous paragraph. Now the association constant for binding to dissolved proteins in Eq. 3 is denoted as K_P . Eq. 3 will contain an additional term, for binding to solidified Matrigel, decreasing the free drug concentration. This term is given as $K_S \times [D]$, as follows from the definition of the association constant K_S . The free drug concentration is then expressed as:

$$[D] = c_D / (1 + H \times V_G / V_A + K_P \times c_P + K_S). \quad (6)$$

The description of the total absorbance is derived in the same way as Eq. 5, with a similar result. The only difference is the presence of K_S in the denominator, as seen in Eq. 6. The absorbance of the DMEM medium is significant and increased by dissolved Matrigel; therefore, we decided to measure the absorbance A_P of the control system that was setup in the same way as the sample, with the omission of the drug. The resulting expression is:

$$A - A_P = [(\varepsilon_D + \varepsilon_{DP} \times K_P \times c_P) \times c_D] / (1 + H \times V_G / V_A + K_P \times c_P + K_S). \quad (7)$$

Equation 7 was used to fit the total absorbance A as a function of total drug and protein concentrations (c_D and c_P) and the absorbance of the protein solution A_P . The fitted parameters were K_S and K_P . The other values (H , ε_{DP} , K_P , ε_D , ε_P) were determined in independent experiments. For non-volatile compounds, $H = 0$.

3. Results and discussion

To maintain consistency and eliminate the problem of the batch-to-batch variability, the same lot of Matrigel was used throughout this study. Phenol red-free Matrigel [31] and serum free medium D-MEM [32] were used in order to minimize interference with UV-Vis absorption of studied compounds used for quantification.

3.1. Kinetics of the solidified layer dissolution

The time-dependent protein release during incubation is a factor that complicates quantification of binding of tested compounds. Washing of the gelled Matrigel with the buffer was tried in the hope that, after elimination of the initial portion of quickly dissolving proteins, the dissolution rate will be minimal. In sequential washing, the first wash contained the largest portion of solidified protein, up to 17%, and the release was continually decreased but remained significant: the washes nos. 3-5 dissolved about 3% each. The washing was abandoned because it resulted in significant protein loss, severe ruggedness of the surface, and deformation of the gelled layer after the first two washes (Fig. 2).

Fig. 2.

Dissolution kinetics of matrix for the dilution ratios 1:3 (■), 1:2.5 (◆) and 1:2 (○). A dilution higher than 1:3 causes fast dissolution of solidified Matrigel and loss of the gel form.

The varying protein content in each wash solution prompted us to examine whether the proteins are released evenly from the whole volume of the solidified layer or mainly from the surface region. We accomplished this using Matrigel layers with the same surface area but differing in thickness. To optimize the solidification times for this situation, different volumes of Matrigel were added to a set of vials and left to gel at 37°C for times ranging between 2 and 6 hrs. Borate buffer was added to each solidified gel preparation and protein release at room temperature was analyzed after 35-minute incubation using the Bradford (1976) assay. The minimal amounts of protein were released from Matrigel preparations with 0.5 mL and 1.5 mL Matrigel and solidification times 2 and 4 hours, respectively.

To evaluate the protein release for layers differing in thickness, three preparations with 0.5, 1.0, and 1.5 mL loads of Matrigel were solidified in the 40-mL flat-bottom vials with internal cross-sectional area of 4.91 cm² for 2, 3, and 4 hours, respectively. The solidified Matrigel was incubated with 2 mL of borate buffer for up to 8 hrs and protein dissolution was monitored. All preparations displayed a time-dependent increase of protein content in the buffer in the

initial phase and leveled-off later on (Fig. 3.). The time needed for the protein release to reach the equilibrium was about 240 minutes for all three preparations. The 1.5-mL preparations showed the largest dissolved protein amount of 377 $\mu\text{g/mL}$, but the lowest fractional protein release (8.7 %). With the decreasing Matrigel volumes, the absolute release decreased and the fractional release increased, reaching 10.6% and 13.4 % for the 1.0-mL and 0.5-mL preparations. The results indicate, in accord with the washing experiments, that the proteins are released from the whole volume of the solidified layer but not homogeneously: more release is observed closer to the surface. Considering the cost of the larger volumes of Matrigel, 0.5-mL preparation was selected for the experiments. The 4-hour incubation with the buffer before the addition of the tested compound was chosen to attain the protein dissolution equilibrium.

3.2. Dissolution of major Matrigel proteins

The Bradford assay [28] determines the total concentration of released proteins. If the dissolved protein has a different composition than the original Matrigel, the binding affinity of small molecules to solidified and dissolved Matrigel might vary. Western Blot analysis was used to examine the content of two major Matrigel components in the dissolved protein fraction. Sample solutions for analysis of laminin-1, collagen IV, and the total dissolved protein were prepared at different times in a similar way as for the measurement of binding of sample compound.

Western Blots of laminin-1 and collagen IV were carried out under reducing conditions. Laminin-1 in the EHS tumor consists of three chains designated as $\alpha 1$ (400 kDa), $\beta 1$ (210 kDa), γ (200 kDa), which are arranged in a cross-shaped structures [33]. Though laminin-1 being a large protein is normally analyzed using 5% separating gel [31], we found that a separating gel containing 7.5% acrylamide worked just fine for 200 kDa bands, representing $\beta 1$ and/or γ chain. The latter two proteins do not separate on the gel and therefore were digitized together. The composition of collagen IV in Matrigel cannot be determined by Western Blot due to anomalous migration on the gel and direct comparison with standards cannot be made [31]. For collagen IV, 125-kDa bands were used to for quantification because of their superior resolution (Fig. 4A.). The band density was converted to percentage of laminin and collagen relative to the specific amounts in original preparation. The Bradford assay [28] was used to characterize the dissolution of total protein (Fig. 4B.).

In terms of the total protein, the plateau was reached after 2 hours with fractional protein release of 16 %. In contrast, the content of both laminin and collagen IV in the solution continuously increased and did not level-off within 6 hours. The fraction of dissolved laminin increased continuously during the monitored period, reaching about 17.4 % after 6 h incubation. Collagen IV kept a steady dissolved fraction of 2-3 % for the first two hours and started dissolving a bit faster later, in accordance with its role as the structural support of basement membrane networks. It seems that proteins other than laminin and collagen IV contribute to early stages of release in the first two hours.

3.3. Quantification of binding

The UV-Vis spectrophotometry was used to determine the equilibrium concentrations of tested drugs in the solution contacting the solidified Matrigel. Absorbance of the aqueous solution is due to drug (D), dissolved Matrigel protein (P), and drug-Matrigel protein complex (DP). For volatile compounds, evaporation affects the distribution in the closed system. The equilibrium is characterized by the association constant to dissolved Matrigel proteins (K_P), the association constant to solidified Matrigel (K_S), and, for volatile compounds, the Henry constant (H). The compounds probably bind to several sites at several proteins; therefore, the association constants K_S and K_P represent the average values. The equations that were used to determine individual parameters by nonlinear regression analysis are listed in Materials, part 2.4.3. If a

drug showed evaporation greater than 3 % during 2-hr incubation, it was classified as volatile and its Henry constant (H) was determined in a separate experiment.

The possibility of using diluted Matrigel to imitate solidified Matrigel in the binding studies was examined. If the results were comparable to binding to solidified Matrigel, this approach would result in significant time and cost savings. The association constants for binding to diluted Matrigel proteins (K_D) were measured in separate experiments for all compounds along with the absorption coefficients of the drug-Matrigel protein complex at appropriate wavelengths. For illustration of the association constant determination for binding to diluted, dissolved and solidified Matrigel (K_D , K_P , and K_S , respectively), 2,2-diphenylethanol and N,N-dimethylaniline were selected as representatives of non-volatile and volatile compounds, respectively.

Evaporation affects the evaluation of other parameters and was examined first. For the volatile compound N,N-dimethylaniline, the kinetics of evaporation was measured as a loss from buffer without Matrigel in closed 40-mL vials. Substantial evaporation occurred in 2 hours and reached equilibrium within 6 hours. The last four time point readings were averaged to give the fraction remaining in solution (94.27 %) which was converted to the Henry constant $H = (3.018 \pm 0.204) \times 10^{-3}$, a dimensionless measure of the evaporation (Fig. 5A.).

The binding constants to diluted Matrigel proteins (K_D) were determined by titration of the compound solution with a solution containing diluted Matrigel proteins. The titration was normally carried out in a 3.5-mL quartz cuvette containing the drug dissolved in 2.4 mL of borate buffer with 1 % of dimethylsulphoxide (DMSO). Diluted Matrigel was added in 5-min time intervals, which were sufficient to establish the binding equilibrium. The titration stopped when the new added volume reached 10 % of the initial volume or when precipitation occurred, whatever happened first. For instance, when titrating 2,2-diphenylethanol, only six additions could be made before the solution became opalescent (data not shown). The fit of Eq. 5 to the N,N-dimethylaniline binding data was satisfactory as characterized by the correlation coefficient $R^2 = 0.997$ (Fig. 4B.). The optimized value of the association constant K_D was $(4.108 \pm 1.874) \times 10^4 \text{ M}^{-1}$.

Determination of the association constants K_P and K_S , characterizing binding to dissolved and solidified Matrigel, respectively, was carried out in one experiment in 40-mL vials. The solidified Matrigel was first incubated with pure buffer for 4 hours to achieve the protein dissolution equilibrium. The compound solution in DMSO (20 μL , 1% DMSO final concentration) was added to the buffer and the incubation continued for two more hours. The top solution was transferred to a cuvette with a Pasteur pipette. The absorbance of the control without drug (A_P) was subtracted from the absorbance of the sample with the drug (A). The difference $A - A_P$ was plotted against the total drug concentration as shown in Fig. 5C. Nonlinear regression according to Eq. 7 resulted in a fit line with $R^2 = 0.991$ for N,N-dimethylaniline. The resulting K_P and K_S were $483 \pm 81 \text{ M}^{-1}$ and 0.546 ± 0.024 , respectively.

3.4. ECM binding vs. physicochemical properties

Ten benzene derivatives were selected based on availability, structural diversity, and physicochemical properties like solubility and lipophilicity (Table 1). Lipophilic chemicals exhibit the tendency to bind to either non-polar environments in membranes which eventually affects their permeation rate through membrane [34] or hydrophobic binding sites in globular proteins like albumin [9,35,36]. The driving forces for nonspecific binding are hydrophobic, electrostatic, and other noncovalent interactions. Nonspecific binding frequently does not depend on exact molecular structures of chemicals and may correlate, in a limited series of compounds, with physicochemical properties like solubility or reference partition coefficients [37].

For all ten compounds, binding to diluted Matrigel proteins was analyzed and the K_D values are summarized in Table 1. The association constants for dissolved (K_P) and solidified Matrigel (K_S) are also given in Table 1, with the exception of benzene for which neither K_P nor K_S were measured because of the problems with evaporation. Benzene evaporates significantly ($H = 31.07$, Table 1) in a 3.5 mL sealed cuvette with the volume ratio V_G/V_A of 0.26 (Eq. 1). In a 40 mL vial with a V_G/V_A of about 20, we were not able to measure K_P and K_S because almost no benzene would remain in solution. We preferred to work with the same buffer volume for all compounds so that the Matrigel protein dissolution remained at about the same level.

Binding to dissolved Matrigel proteins, described by the association constant K_P , correlates well on the bi-logarithmic scale with lipophilicity (slope = 0.698 ± 0.071 , intercept = 1.231 ± 0.160 , $R^2 = 0.918$). A moderate correlation was observed between the binding constant to solidified Matrigel (K_S) and $\log P$ (slope = 0.370 ± 0.025 , intercept = -1.053 ± 0.065 , $R^2 = 0.827$), possibly due to a narrower range of the K_S values. The association constant for diluted Matrigel proteins (K_D) demonstrated the weakest linear bilogarithmic correlation with P (slope = 1.195 ± 0.066 , intercept = 0.574 ± 0.142 , $R^2 = 0.763$). These relationships can provide rough estimates of the binding of simple benzene derivatives to Matrigel proteins in different forms. However, the scatter of data indicates that the trends are approximate and may not hold for drugs with more complicated structures. This phenomenon requires further examination.

The solidified Matrigel is the sole source of dissolved proteins. In spite of different compositions of the dissolved proteins and the solidified layer, both the K_P and K_S values exhibit a tendency to increase with lipophilicity, albeit with different slopes (Fig. 6). How mutually dependent are these two parameters? Linear regression of the data in Table 1 revealed moderate correlation ($R^2 = 0.630$) between $\log K_P$ and $\log K_S$, suggesting that protein composition plays a role in binding. As shown by Western Blot analysis, the dissolved fraction contains more laminin and the solidified Matrigel is richer in collagen IV.

We measured the binding to diluted Matrigel (K_D) in the hope that these direct titrations with the protein solution could be used in lieu of the lengthy experiments with solidified Matrigel providing the K_P and K_S values, especially for a fast screening of drug candidates. The results did not confirm our expectations. The correlations of the association constants for dissolved and solidified Matrigel (K_P and K_S , respectively) with the association constants for the diluted Matrigel (K_D) are less than satisfactory for this purpose: on the bi-logarithmic scale, $R^2 = 0.716$ and 0.543 , respectively. Consequently, the titrations with diluted Matrigel cannot be used to replace more tedious experiments with solidified Matrigel.

The described method can help in the development of inhibitors, effectors, and other drug candidates acting in the ECM, with optimization of bioavailability in tissues. Excessive ECM binding can compromise the activities by decreasing available concentrations around the receptors. On the other hand, a moderate ECM binding can be beneficial for creation of a depot that will buffer the influence of elimination and maintain the concentrations at desired levels for a certain time. Non-specific binding is normally expected for small compounds binding to macromolecules. The dependencies of binding to various Matrigel forms on lipophilicity of tested chemicals showed clear trends but, in general, significant scatter even for the studied simple compounds. This fact indicates that lipophilicity is not the most suitable predictor of binding to extracellular matrix and either more predictors are required or a more sophisticated, structure-based approach is necessary to generate the correlations that could be used in prediction of binding to ECM for diverse drug structures.

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Abbreviations

A, A_P	absorbance of the sample and the control, respectively
c_A, c_D, c_P	concentration in aqueous phase, total, bound to dissolved proteins, respectively
D, D_G, D_S	drug free, evaporated, bound to solidified Matrigel, respectively
DP	drug, drug protein complex
DMSO	dimethylsulphoxide
ECM	extracellular matrix
$\varepsilon_D, \varepsilon_{DP}, \varepsilon_P$	absorption coefficient of drug, drug-protein complex, protein, respectively
H	Henry constant
K_D, K_P, K_S	the association constants for binding to diluted, dissolved, and solidified Matrigel, respectively
n_A, n_D	amount in aqueous phase, total, respectively
O.D.	outside diameter
P	protein
P	1-octanol/water partition coefficient
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TRIS	tris-(hydroxymethyl)-aminomethane
V_A, V_G	volume of the aqueous and gas phases, respectively

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Fig. 1.

A scheme of interactions of drug molecules D with the solidified extracellular matrix (D_S , the bottom layer) and with dissolved extracellular matrix proteins in the buffer (DP , the middle layer). Evaporation of volatile compounds is also included. The parameters characterizing individual equilibria are shown. The association constant for binding to dissolved proteins is termed K_P in experiments with solidified Matrigel and K_D in experiments with diluted Matrigel where no solidified Matrigel layer is present.

Fig. 3.
Effect of the layer thickness on matrix dissolution: absolute (top) and fractional protein release (bottom) for 0.5 mL (■), 1.0 mL (○), and 1.5 mL (◆) of Matrigel.

Fig. 4.

Release of proteins from solidified Matrigel. A: Western Blot for laminin-1 (top); and collagen IV (bottom); (left →right) standard laminin (0.62 mg) or standard collagen IV (0.96 mg), diluted Matrigel (115 µg/mL), protein released after 0.5, 1, 2, 4, and 6 hours of incubation of solidified Matrigel with buffer; B: Calculated fractional release of the whole protein by Bradford assay (white), fractional release of laminin (hatched) and collagen IV (black) relative to their total concentrations in time.

Fig. 5.

Kinetics of evaporation (A) and the determination of the association constants of N,N-dimethylaniline to diluted (B), as well as dissolved and solidified Matrigel (C). A: The last three or four points were used to determine the Henry constant using Eq. 1. B: Absorbances were plotted against the total protein concentration for a titration by diluted Matrigel solution. C: Absorption differences between the samples and controls were plotted against the total drug concentration. For B and C, the wavelengths from 250 to 270 nm in 5-nm intervals were used. Data in B and C were fitted simultaneously to Eqs. 5 (B) and 7 (C).

Fig. 6. The association constants for binding to diluted, dissolved and solidified Matrigel, K_D (■), K_P (○), and K_S (◆), respectively, as dependent on the 1-octanol/water partition coefficients P on bi-logarithmic scale. The lines correspond to the correlations described in the text.

Studied benzene derivatives, their 1-octanol/water partition coefficients P , the Henry constants H , and binding affinities to diluted (K_D), dissolved (K_P), and solidified Matrigel (K_S). The H and K_S values are dimensionless. The K_D and K_P values are in M^{-1} .

Table 1

Compound	$\log P$	H	$\log K_D$	$\log K_P$	$\log K_S$
benzene	2.13	31.07 ± 0.10	3.100 ± 0.020	-	-
phenol	1.46	-	2.121 ± 0.113	2.451 ± 0.155	-0.479 ± 0.046
aniline	0.90	$(2.834 \pm 0.770) \times 10^{-3}$	1.931 ± 0.170	1.753 ± 0.164	-0.556 ± 0.094
n,n-dimethyl aniline	2.31	$(3.018 \pm 0.204) \times 10^{-3}$	4.614 ± 0.198	2.684 ± 0.073	-0.263 ± 0.019
4-bromophenol	2.59	-	3.830 ± 0.130	3.399 ± 0.233	-0.158 ± 0.183
p-nitroaniline	1.39	-	2.566 ± 0.207	2.096 ± 0.130	-0.951 ± 0.098
2-nitrotoluene	2.30	-	3.776 ± 0.140	2.895 ± 0.240	-0.092 ± 0.050
2,2-diphenylethanol	2.77	-	4.535 ± 0.128	3.409 ± 0.193	-0.017 ± 0.011
4-chloro-3-methylphenol	3.10	-	3.647 ± 0.207	3.223 ± 0.168	-0.201 ± 0.109
2,4,5-trichloroaniline	3.45	$(8.214 \pm 0.679) \times 10^{-2}$	4.368 ± 0.157	3.448 ± 0.227	-0.275 ± 0.164