Trends in Analytical Chemistry

Human artificial membranes in (bio)analytical science: Potential for in vitro prediction of intestinal absorption-A review

--Manuscript Draft--

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Abstract:	Artificial membranes for investigation of the human absorption of target organic compounds are aimed at mimicking the interactions occurring within the lipid membrane. In this review, we will differentiate biomimetic platforms based on static and dynamic modes. Parallel artificial membrane permeation assays are the most common approaches for static mode while in dynamic modes, there is a plethora of bioanalytical techniques such as immobilized artificial membrane chromatography, biopartitioning micellar chromatography or immobilized plasma protein chromatography. In any case, all of the dynamic approaches capitalize the use of the chromatographic factors to predict instetinal absorption. However, improvements in the fabrication of novel sorptive materials or the development of innovative techniques to enhance the prediction of permeability has been left in the background. For this reason, this review covers the current state-of-the-art of immobilized artificial membranes in bioanalytical science with particular focus on new materials and techniques reported from 2015 to mid-2021.
Suggested Reviewers:	Frederik Hansen University of Oslo: Universitetet i Oslo f.a.hansen@farmasi.uio.no He has experience in the preparation of membranes and analysis of compounds in biological matrices
	Fotios Tsopelas University of Athens: Ethniko kai Kapodistriako Panepistemio Athenon ftsop@central.ntua.gr He has experience in chromatographic techniques for the evaluation of pharmacokinetic properties (human oral absorption, protein binding) of candidate drugs and ecotoxicological profile of pollutants.
	Dana Moravcová Institute of Analytical Chemistry CAS: Ustav analyticke chemie Akademie Ved Ceske Republiky moravcova@iach.cz She has experienece in the preparation of novel systems for IAM chromatography
	Dietmar Knopp Technical University of Munich: Technische Universitat Munchen dietmar.knopp@mytum.de He has experience in the study of biological processes
	Elena Sánchez-López Leiden University Medical Center: Leids Universitair Medisch Centrum

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	E.Sanchez_Lopez@lumc.nl She has experience in the study of biological interactions
	Rafel Lucena-Rodríguez University of Cordoba: Universidad de Cordoba rafael.lucena@uco.es He has experience in the preparation of novel materials
Opposed Reviewers:	
Response to Reviewers:	

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15th September 2021

Dear Prof. Stig Pedersen-Bjergaard,

Enclosed please find the revised manuscript with the new title "*Human artificial membranes in (bio)analytical science: Potential for in vitro prediction of intestinal absorption-A review*" for potential publication in the special issue "*New extraction phases and chemistries in analytical chemistry*" in the journal TrAC-Trends in Analytical Chemistry.

We appreciate very much the insightful suggestions from the two reviewers, and we are very pleased with their positive evaluation and the recommendation for publication following the minor revision. Every improvement and corrections of the manuscript suggested by the referees has been addressed point by point in the revised manuscript and in a new file namely "response to reviewers".

We hope to receive a positive review from you and from the peer-review experts.

Yours sincerely

Enrique Javier Carrasco-Correa

Special issue – Trends in Analytical Chemistry

New extraction phases and chemistries in analytical chemistry

Editor:
Stig Pedersen-Bjergaard

Suggested guest editors:
J.L. Anderson, K.H. Row, Y. Yamini, B. Sellergren, G. Ouyang, M. Miro

Idea
Each of the six guest editors contribute with a review article, and invites two or three additional reviews in the area of "new extraction phases and chemistries in analytical chemistry". In total, we will invite 18-24 review articles. The guest editors are very experienced in the field, and as team, we can put together a very interesting collection of papers.

Content
Initially we will invite the following guest editors and ask them to write about the following hot topics:

Ionic liquids
J.L. Anderson (h-index 42) - andersoj@iastate.edu

Deep eutectic solvents
K.H. Row (h-index 36) - rowkho@inha.ac.kr

Nanostructured supramolecular solvents
Y. Yamini (h-index 61) - yyamini@modares.ac.ir

Molecularly imprinted polymers
B. Sellergren (h-index 61) - borje.sellergren@mau.se

Metal-organic frameworks
G. Ouyang (h-index 43) - cesoygf@mail.sysu.edu.cn

Artificial Immobilized membranes in (bio) analytical sciences
M. Miro (h-index 39) - manuel.miro@uib.es

Examples of other topics that guest editor invitations might cover are:
Covalent organic frameworks
Magnetic sorbents
Nanoparticles
Layered double hydroxides
Cyclodextrin-based sorbents
Carbon nanotubes
Sol-gel materials
Restricted access materials
Immunosorbents
Mixed-mode ion-exchange polymeric sorbents

Critical eye

We kindly ask all authors to address the following critical questions as part of their reviews:

- How does the new extraction phase work (fundamentals, molecular interactions)?
- What are the top 10 best papers from 2017-2020 using the new extraction phase?
- What are the main advantages of the new extraction phase?
- What are the main disadvantages of the new extraction phase?
- What are the potential killer applications with the new extraction phase, and are these true killer applications?
- The new extraction phases…
    - …commercially available?
    - …automation?
    - …potential for routine use?
    - …potential for measurements not feasible with current methods?
- Future research with the new extraction phase, what are the main directions?

Time schedule

Invitation of guest editors	May 20, 2020
Invitation of additional reviews	July 1, 2020
Manuscript submission deadline	January 1, 2021
Final acceptance deadline	March 15, 2021

Compliance with author guidelines

We kindly ask authors to read author guidelines, and comply with these during manuscript preparation. During manuscript submission, authors should select Stig Pedersen-Bjergaard as handling editor.

Introductory text to special issue

Sample preparation is a very active research area in analytical chemistry. Major incentives for this are to increase selectivity, clean up, enrichment, sample throughput, compatibility with analytical instrumentation, speed, simplicity, automation, to reduce the consumption of chemicals and reagents, and to facilitate soft extraction. Recently, we highlighted microextraction technologies in a virtual special issue of Trends in Analytical Chemistry, and a collection of reviews discussed different approaches to solid-phase microextraction (SPME) and liquid-phase microextraction (LPME). SPME and LPME are similar to classical solid-phase extraction (SPE) and liquid-liquid extraction (LLE), but with the former techniques, the extraction phase is downscaled. In parallel to research on different microextraction systems, new type of extraction phases and chemistries are developed and evaluated. These are new types of sorbents, such as molecularly imprinted polymers and metal organic frameworks, and new type of liquids such as ionic liquids and deep eutectic solvents. Scientists often implement new sorbents and liquids in microextraction systems and microfluidics, but also in more classical type extraction systems. In the current virtual special issue of Trends in Analytical Chemistry, we have asked leading scientists to review and critically discuss some of the new extraction phases and chemistries. We have challenged the authors, and asked them to discuss critically the potential for replacing existing methods or for development of killer applications where existing methods are insufficient.

Response to reviewers

Reviewer #1: The review refers to the application of biomimetic chromatography and relevant assays (e.g. PAMPA) to model biological purposes. The article is timely, well-written and it can be a good addition to the existing literature. In order to increase the impact of this work, my detailed comments are appended below:

Answer: First of all, thank you for all your insightful suggestions to improve the quality of this manuscript and to give us the opportunity to revise it.

1) Numbering of paragraph is not correct. For example, introduction is not numbered, the next paragraph is numbered 1 and 1.1, next paragraph is numbered 1.2. and 1.2.1. (if a paragraph is numbered 1.2.1. the reader searches for paragraph 1.2.2), the next paragraph is numbered 2.2.2 (!), etc. Please pay attention to the numbering of paragraphs.

Answer: There was a confusion with the numbering of the paragraphs, and this was occasioned during the building of the pdf file. The numbering of paragraphs was corrected in the revised manuscript.

2) Title: I suggest authors to rephrase the title, as the study refers to ADME properties. Authors should avoid the term "bioavailability" (see comment below) and replace it by "ADME properties" or "model biological processes", etc.

Answer: As suggested by the reviewer (also along comment #3), a new title for the manuscript has been proposed: "Human artificial membranes in (bio)analytical science: Potential for in vitro prediction of intestinal absorption-A review"

3) Bioavailability and human oral absorption/ intestinal absorption: The main audience of this journal is Analytical Chemists, who are NOT likely to be familiar with bioavailability and oral absorption. Therefore, my major suggestion is to clarify these terms as much as possible and to use the appropriate one. For example, at line 53, as it is written, a reader may be misled to a conclusion that bioavailability is the same thing with human absorption. Furthermore, in lines 346-347 the term of "human intestinal absorption" is used, while in lines 66, 191 and 475 authors prefer the word "bioavailability". However, bioavailability is something different than oral (intestinal) absorption, and it refers to the percentage of an administered dose of a xenobiotic that reaches the systemic circulation. Bioavailability depends both on intestinal absorption and on first-pass metabolism. Therefore, bioavailability is equal or lower than oral absorption, depending on the extension of the first-pass effect. However, all described assays can not really predict the first-pass metabolism, but only intestinal absorption. Therefore, I suggest authors to use the term "(human) oral absorption" or "intestinal absorption", because this process is the one that can be simulated.

Answer: As recommended by the referee, potential misleading statements concerning the terms "bioavailability" and "human oral absorption/intestinal absorption" have been eliminated. Therefore, along the manuscript, the term bioavailability has been changed by "intestinal absorption (IA)" .

4) Line 73: Replace "octanol-water conditions" with "octanol-water system".

Answer: The term "octanol-water conditions" has been changed by "octanol-water system".

5) Line 74: Replace "..predict the lipophilicity.." with "..to express the lipophilicity".

Answer: The term "..predict the lipophilicity.." has been changed by "..to express the lipophilicity".

6) Lines 105-106, 124-126, 397-400: Authors use the term "biomimetic liquid chromatography" to describe HSA and AGP stationary phases. I do not fully agree with this term. All chromatographic techniques described in this review (IAM, micellar chromatography and HSA/AGP) can be characterized as biomimetic. I suggest authors to use the term "Immobilized Plasma Protein Chromatography" which is more accurate.

Answer: As suggested, the term BLC has been changed by "Immobilized Plasma Protein Chromatography (IPPC)" along the revised manuscript.

7) Paragraph 1.2.1. and Table 1: Two comments can be added: (a) For human oral absorption (intestinal absorption) experiments should be carried out at a certain range of pH due to the pH gradient of the gastrointestinal tract (ideally between 2.0-8.0) and the maximum retention should be considered (https://doi.org/10.1016/j.ejps.2015.09.020 and https://doi.org/10.1016/j.ijpharm.2008.04.025), (b) The use of MS as a detector of the chromatographic system offers the opportunity to inject mixtures of the compounds under investigation (and not to inject compounds one by one) and therefore the screening process speeds up. However, in this case a compatible eluent with MS should be selected (e.g. PBS can not be used), which can also model biological fluids.

Answer: In the revised manuscript, a new paragraph (lines 311-324, pages 19-20) has been added to include these valuable comments for future researchers that want to explore the possibility to prepare novel systems for IAM chromatography.

8) Lines 318-326: (a) Comparison of IAM with biopartitioning micellar chromatography: IAM also predicts %HOA associated with passive diffusion. If the underlying mechanism is other (e.g. via paracellular route in the case of small molecules, active transport), large deviations can be observed, (b) A sentence to explain the biomimetics performed by BMC can be added (Due to the hydrophilic/hydrophobic character of surfactants the modified stationary phase structurally mimics the ordered array of the hydrocarbon chains in membranes as well as the polar membrane regions), (c) A double equilibrium in the case of BMC exists.

Answer: A more detailed information about BMC has been included in the revised manuscript (lines 330-339, page 20) to convey the three main ideas given by the referee.

9) Lines 385-395: Evaluation of BMC: Some comments can be added, such as its advantage to simulate simultaneously a number of pharmacokinetic properties with one only measurement, its low cost and flexibility (e.g. can be used with combinations of surfactants and stationary phases), but it can not be used in gradient conditions (the required time for measurements increases), see: last paragraph of conclusions in https://doi.org/10.1016/j.chroma.2020.461027

Answer: As suggested, some comments based on of the JCA paper by Tsopelas et al. (added to the manuscript, reference 70) has been included in the revised manuscript (lines 401-406 and page 26).

10) Line 409: Replace "HAS" with "HSA".

Answer: The typo was corrected in the revised manuscript.

11) Line 412: Replace "materias" with "materials".

Answer: The typowas corrected in the revised manuscript.

12) Paragraph 2.2.3.: In the end of this paragraph, a brief discussion/ evaluation of immobilized plasma protein chromatography and its perspectives can be added.

Answer: As recommended, a brief discussion, including the future perspective of this topic has been added to the revised manuscript (lines 449-456, page 28)

13) Lines 462-483: Some sentences can be omitted (e.g. 462-463, some results concerning R2, etc). Note that the part conclusions is not an abstract. Conclusions, current trends, perspective and future expectations as well as limitations should be discussed.

Answer: The conclusions have been revised and sentences regarding specific results have been removed.

Reviewer #2: Human absorption, distribution, metabolism and excretion are biological processes that involve several organs, finally determining the bioavailability of a compound, i.e., its levels in tissues. Animal testing (in vivo approaches) still represents a gold standard, especially in pre-clinics (drug testing). However, in vivo models are generally time-consuming, expensive and labor-intensive. Therefore, there is a need to develop alternative in vitro platforms for application in bioavailability studies. Over the last decades, there was published a multitude of articles that report, e.g., about bioavailability and toxicity of different compounds using a variety of models and distinct experimental conditions. On the contrary, the number of related review articles highlighting benefits and drawbacks of tested techniques is rare.

This nicely prepared review focuses on the current state-of-the-art of artificial biomimetic membranes (bio-membrane surrogates) in bioanalytical science to improve bioavailability predictions with special emphasis on the fabrication of new innovative materials and techniques: The authors covered the period from 2015 up to mid-2021. After give attention to static (batch-wise) cell-free artificial membranes for permeability studies, main emphasis was put on dynamic (chromatographic) biomimetic systems, incl. immobilized artificial membrane (IAM) chromatography, bio-partitioning micellar chromatography (BMC) and biomimetic liquid chromatography (BLC) etc. Obviously, the highly interesting development of smart materials involving nanomaterials in combination with membrane surrogates is still a vision and not introduced into practice yet.

Answer: Thank you for all your valuable comments. We fully agree with you thatthe introduction of new materials incorporating artificial membranes is still on the beginning and only few authors tried to prepare novel stationary phases or compounds to improve the in vivo data prediction. However, in our opinion, these novel materials could led to a new dawn in the preparation of biomimetic phases and techniques to improve the data obtained by in vitro technologies.

Special comments

The authors should check and correct numbering of sections. After section 1.2.1. (line 204 on page 9) the section 2.2.2. (line 312 on page 18) follows.

Answer: During the building the pdf file, the numbering of paragraphs was not continuous and, therefore, it was corrected in the revised manuscript.

Page 8, line 171: Abbreviation 'AMI' should be used (not AIM).

Answer: The typo has been corrected in the revised manuscript.

Table 1, page 12: Cited reference '37' is from year 2014. It interferes with covered period (2015-2021) as was stated by the authors on line 47 on page 2.

Answer: It is true that the reference 37 is of October 2014. However, we think that the modification of the stationary phase with sphingomyelin is an interesting alternative that should be included in the table for inspiring potential practitioners.

Page 13, Table 1, second column: 'drugs' should be spelled with a capital letter (Drugs).

Page 15, Table 1, second column: Correct 'Psychopharmaca' (not: Psychopharmacs').

Page 18, line 317: I suppose it should be 'adsorbed' (not: 'absorbed').

Page 24, lines 389, and 390: Please correct 'MEKC' (not: MECK').

Page 25, line 409: I suppose the authors mean 'HSA' (not: 'HAS').

Page 27, line 457 and page 28, line 486: Please correct 'BMEKC' (not: 'BMECK').

Page 29, line 519: Correct journal abbreviation is 'J. Membr. Sci.'. It should be corrected.

Answer: All these typos have been corrected in the revised manuscript.

Highlights:

-Membrane surrogates in analytical science

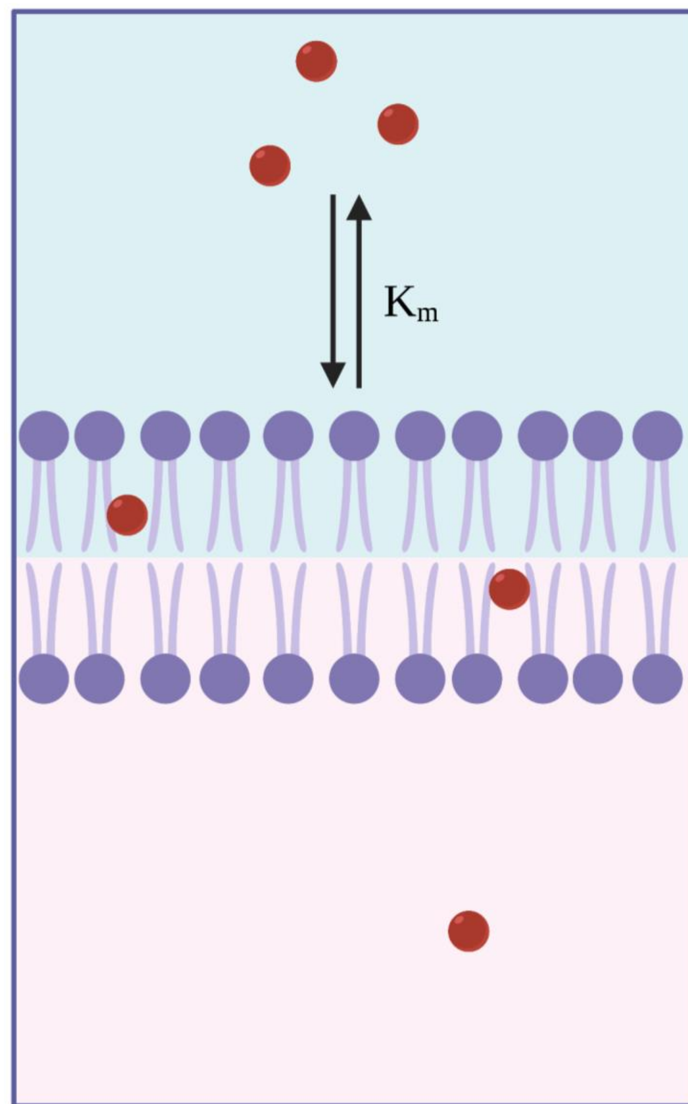
-Role of static and dynamic systems in bioavailability assays

-Overview of parallel artificial membrane permeability assays

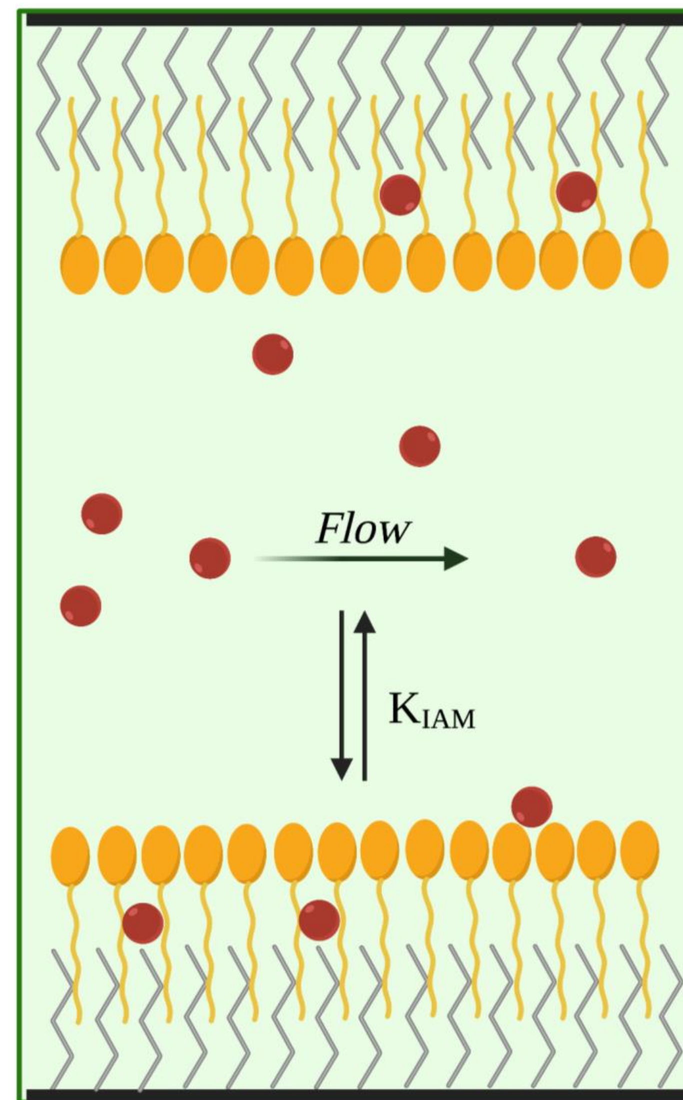
-Overview of immobilized artificial membrane chromatography, biopartitioning micellar chromatography and biomimetic liquid chromatography

-Trends and selected applications reported in the literature since 2015

Graphical Abstract



Static mode



Dynamic mode

Graphical Abstract – Carrasco-Correa *et al.*

Manuscript File control changes
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1 Human ~~Artificial~~ artificial membranes in (bio)analytical
2 science: Potential for *in vitro* ~~bioavailability studies~~prediction
3 of intestinal absorption-A review

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24 **Keywords:** Artificial membranes; biomimetic; parallel artificial membranes;
25 immobilized artificial membrane chromatography; biopartitioning micellar
26 chromaotography; biomimetic liquid chromatography; ~~bioavailability~~intestinal
27 absorption.

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Abstract

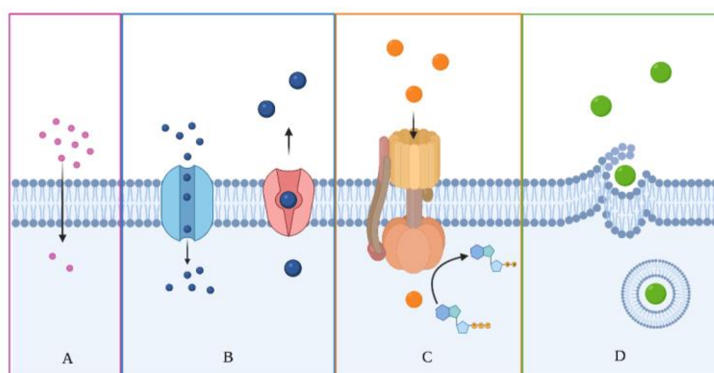
Artificial membranes for investigation of the human absorption (oral, dermal or respiratory) of target organic compounds ~~(bioavailability)~~ are aimed at mimicking the interactions occurring within the lipid membrane. Biomolecules such as proteins are also integral components of the lipid membranes and play a pivotal role towards ~~bioavailability~~ intestinal absorption ~~and permeability of organic compounds and~~ understanding the complex mechanisms of human absorption~~ (oral, dermal or respiratory)~~. In this review, we will differentiate biomimetic platforms based on static (batchwise) and dynamic modes. In the former, a synthetic membrane placed between two phases (donor and acceptor) mimics a given biological system to study permeability. Parallel artificial membrane permeation assays are the most common approaches for static mode. As to dynamic modes, there is a plethora of bioanalytical techniques such as immobilized artificial membrane chromatography, biopartitioning micellar chromatography or ~~biomimetic liquid~~immobilized plasma protein chromatography. In any case, all of the dynamic approaches capitalize upon analytical separation techniques such as liquid chromatography and the use of the chromatographic factors to predict permeability and other bioparameters. However, improvements in the fabrication of novel sorptive materials or the development of innovative techniques/approaches to enhance the prediction capability of permeability by simulated membranes has been left in the background. For this reason, this review covers the current state-of-the-art of immobilized artificial membranes in bioanalytical science with particular focus on new materials and techniques reported from 2015 to mid-2021. Future perspectives related to the fabrication of innovative artificial membranes for *in vitro* ~~bioavailability~~ intestinal absorption studies have been highlighted so as to encourage fundamental studies in this research area.

1. Introduction

Human absorption ~~(bioavailability)~~ refers to a pharmacokinetic process on the basis of which a given amount of a target compound is able to pass from external sources (oral, dermal or respiratory) through cell membranes and, therefore, enter into a living organism [1,2]. For accurate assessment of the human absorption, the variety of potential interactions between the target species and the cell plasmatic membrane including dipole-dipole, hydrogen bond donor/acceptor, London, cation-π and electrostatic interactions need to be thoroughly studied, yet this is a very complex process that is dominated by the occurrence of different biomolecules: lipids, proteins, and polysaccharides, among others [3]. In addition, the knowledge of the absorption conditions (pH, temperature, fluid composition, etc.) is necessary because might affect the lipophilic nature of the target compound. In this sense, insight into the human compartment from which the target compound is going to be absorbed is particularly relevant because, for example, the pH in the gastric fluid (1.0-1.4) differs substantially from that of the plasma (*ca.* 7.4) or that of the small intestine (6.5-8.5) [3] and thus the ~~bioavailability~~ intestinal absorption (IA) of ionizable compounds might be significantly altered.

The pathways for compounds (drugs, nutrients, unwanted xenobiotics, etc.) to pass through the lipidic membrane are severalfold and are deeply discussed in previous reviews [3,4] as summarized in Fig. 1. Briefly, the absorption processes could be divided in: (i) passive diffusion in which a net movement of the compound from one side of the membrane to the other is related to the concentration gradient (Fick's law) (Fig. 1A). The partition coefficient (P) in octanol/water ~~conditions~~ system is the most common parameter to ~~predict~~ express the lipophilicity of a chemical, and therefore the ability to be transported by diffusive transport; (ii) protein-mediated transfer that uses membrane proteins as carriers to generate pathways through the lipid membrane (facilitated

78 diffusion, see Fig. 1B); (iii) active transport that allows the movement of molecules
79 against the concentration gradient, polar repulsion, or other resistive forces using
80 membrane proteins and employing energy (adenosine triphosphate, ATP) (see Fig. 1C);
81 (iv) endocytosis-facilitated process that consists of the transport of large molecules
82 (proteins, polysaccharides, etc.) by engulfment of the compound by the cell membrane
83 itself (see Fig. 1D).



84

85 **Fig. 1.** Scheme of the different pathways for endogenous and xenobiotic compounds to
86 pass through the lipid membrane. Passive diffusion (A); facilitated diffusion (B); active
87 transport (C); endocytosis-facilitated process (D). Created with BioRender.com.

88 Up to date, a vast amount of the literature is focused on investigating the interactions
89 between drugs and the lipid membrane and also with membrane proteins in order to
90 elucidate different biologically-relevant parameters, such as Log $P_{oct/water}$ (in neutral, P^N,
91 or ionized, *D*), Log *BB* (blood-brain), Log P_{eff} (effective intestinal/Jejeunal permeability)
92 or protein binding, among others, as summarized in recent review articles [3,5,6]. Log
93 BB is an important parameter that is defined as the logarithm of the ratio of the
94 concentrations of a target compound in the brain and in the blood under equilibrium
95 conditions. This bioparameter gives insight into the blood-brain barrier (BBB)

permeability. For *in vivo* measurements, the concentration of the target compound is analyzed in the brain and blood of a rat previously administrated with the compound [7]. The Log P_{eff} is the logarithm of the *in vivo* human effective permeability of the target compound in a specific zone of the intestine (duodenum, jejunum or ileum) and can be calculated by measuring the permeation rate of the target compound during intestinal perfusion [8]. Although the *in vivo* approaches are the most accurate methods to predict bioparameters, the use of *in vitro* cell-free methodologies exploiting QSAR (quantitative structure-activity relationship) calculations have attracted the interest of researchers over the last few years. To this end, artificial biomimetic membranes (ABM) using cell-free permeation systems [9] in batchwise mode, and immobilized artificial membrane (IAM) chromatography, biopartitioning micellar chromatography (BMC) and ~~biomimetic liquid chromatography (BLC)~~immobilized plasma protein chromatography (IPPC) in dynamic mode have emerged as appealing *in vitro* counterparts. With respect to ABM methods, the parallel artificial membrane permeability assay (PAMPA) is commonly reported in the literature, although other alternatives, such as the phospholipid vesicle-based permeation assay (PVPA), and the Permeapad® and the artificial membrane insert (AMI) systems are worth mentioning [10]. In the original PAMPA, egg lecithin containing a mixture of phospholipids (phosphatidyl choline, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI) as major cell membrane components, dissolved in n-dodecane, is employed to mimic the lipid membrane of eukaryote cells [11]. For this purpose, a polyvinylidene fluoride (PVDF) filter is soaked in the lipid solution and placed between two liquids, the donor phase and the acceptor phase until reaching steady state. However, this ABM method underestimates the fraction of target species absorbed due to the absence of other key interactions occurring in biological systems. Therefore, dynamic variants that are focused on separation techniques, mainly chromatography, namely,

IAM, BMC and ~~BLC~~ IPPC are gaining momentum [11]. In short, lipid monolayers based on phospholipids are in IAM chromatography covalently linked to silica or monolithic stationary phases. The retention factors of target compounds using IAM columns in liquid chromatography are related to bioparameters [3]. In BMC, micellar pseudostationary phases mimicking the liposome structure are adopted [12]. ~~BLC~~ IPPC measures the binding of target species with proteins in the blood stream or membrane surfaces using stationary phases containing immobilized human serum albumin (HSA) or alpha-1-acid glycoprotein (AGP) [5], respectively.

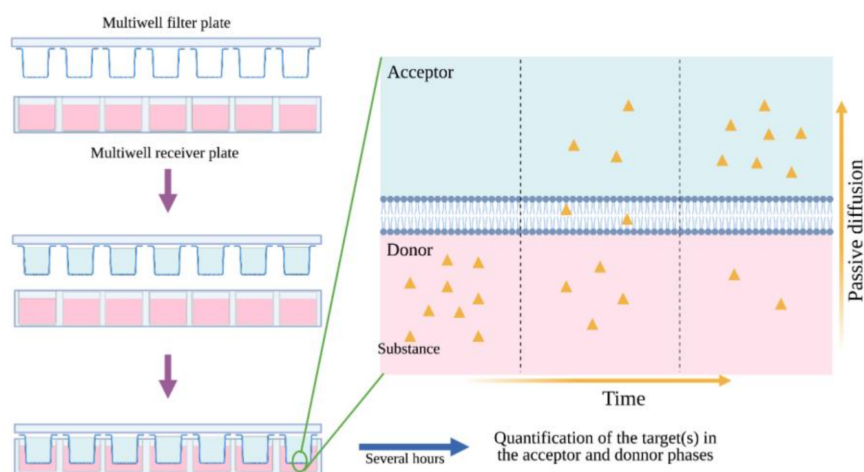
This review is aimed at critically assessing *in vitro* chromatographic and static methods mimicking biological membranes (lipid bilayers) that have been recently resorted to the prediction of bioparameters, with emphasis on innovations of chromatographic materials and biorelevant cell surrogates, and their possibilities to act as predictors of the ~~bioavailability~~ IA of drugs and pollutants.

2. Cell-free artificial membranes for permeability studies

2.1. Static (batchwise) systems: Artificial biomimetic membranes

As indicated above, PAMPA is the most common ABM cell-free methodology to explore the *in vitro* permeability/~~bioavailability~~ IA of drugs and contaminants in the human organism. A scheme of PAMPA is shown in Fig. 2. The simplicity of the procedure and the flexibility for incorporating varied lipid bilayers, including real and synthetic membranes, have made it a very attractive alternative to researchers. Readers are referred to comprehensive articles on the trends in PAMPA methodologies exploring distinct membranes and/or using chemometrics to build suitable models [9,13,14]. In most cases, the literature studies are focused on the prediction of pharmacokinetic parameters and

145 studying the permeability of varied targets through biomembrane surrogates [15–18].
146 Nevertheless, PAMPA-related synthetic membranes have been limited so far to PVDF
147 supports coated with varied phospholipid constituents and oil membranes for
148 gastrointestinal absorption, BBB and skin [10,13]. On the other hand, other ABM
149 methodologies have been proposed to obtain more representative models of the human
150 barrier, such as PVPA, Permeapad® and AMI systems [10], as explained below.



151

152 **Fig. 2.** Scheme of the PAMPA procedure and magnification of the passive diffusion of
153 targets through lipid bilayers. Created with BioRender.com.

154 PVPA is an ABM approach that consists of incubating a liposome-laden filter support
155 that will act as a barrier mimicking the phospholipid bilayer of the intestinal cell
156 membrane [19,20]. By changing the membrane composition other specific human organs
157 could be easily simulated [21]. Recently, the incorporation of the mucus layer has been
158 introduced as an interesting alternative to standard PVPA [22–24]. This modification
159 relies on the fact that the mucus layer is the first barrier that the targets will need to cross
160 for absorption through epithelial tissues and is mimicking all mucosal surfaces in the

human body. For example, Calvo-Lerma *et al.* [25] combined mucus-PVPA with the *in vitro* intestinal lipolysis model, which simulates physiological gastrointestinal conditions, to study nutrient hydrolysis. In this case, the authors evaluate the permeation *in vivo* (measured as the so-called area under the curve) of fenofibrate in self-nanoemulsifying drug delivery systems. In this combined system, the amount of drug solubilized over time during lipolysis did not correlate with the *in vivo* absorption ($R^2 < 0.4$). However, the permeated amount using the mucus-PVPA methodology after lipolysis did have a strong correlation with the *in vivo* data ($R^2 = 0.995$) while the mucus-PVPA permeation in the absence of lipolysis was also well correlated with *in vivo* permeation ($R^2 = 0.926$). The main conclusion of this work is that the use of mucus-PVPA in combination with gastrointestinal fluids might offer better simulation of the human absorption conditions.

Permeapad® is another ~~AIM~~ AMI based on the use of PC immobilized between two barriers so as to avoid leaking. The PC forms lipid crystals which in the presence of water swell and build a tightly packed layer of spheroids with lipid bilayers intercalated with water layers as cellular membrane surrogates. Generally, Permeapad® is used in combination with 96-well plate, disks for side-by-side chambers or Franz diffusion cells [10]. Generally, Permeapad® is aimed at evaluating drug permeability [26–28] but no innovation regarding the membrane surrogate has been performed since the first Permeapad® model launched in 2015 [29]. Only modifications concerning the increase of the interfacial area-to-donor-volume-ratio [30] have been reported for improving the correlation with rat ~~bioavailability~~ IA against those obtained with traditional permeation systems (side-by-side systems and Caco-2-cell membranes).

AMIs are (phospho)lipid-free permeation systems consisting of a regenerated cellulose membrane barrier with a given molecular mass cut-off that is placed between two plastic rings [31]. For example, a reasonable correlation was observed for poorly water-soluble

drugs dissolved in simulated/human intestinal fluids against the standard Caco-2 absorption system [32]. Also, AMI can be modified with a mucus layer for a better simulation of physiological conditions [33]. In brief, AMI is a cost-effective high-throughput approach to investigate passive permeation. Yet, innovations concerning AMI are still to come.

Another *in vitro* testing assay commonly adopted in pharmacological applications for mimicking passive ~~bioavailability~~ IA is the so-called Franz-cell system [34]. Here, the donor and receptor compartments are separated by the animal model membrane with the stratum corneum facing the donor compartment. Franz-cell permeation systems however are prone to low-reproducibility, changing of the cell model is required depending on the release kinetics, and the use of the instrument is not user-friendly compared to PAMPA systems. In the literature, the main applicability of Franz-cell devices relates to skin permeation, rather than gastrointestinal/BBB transfer. In order to avoid ethical issues associated to excised human or animal skin, replacement of those by synthetic membranes such as Strat-M™ have been proposed as an interesting alternative [35].

2.2. Dynamic biomimetic systems

2.2.1. Immobilized artificial membrane (IAM) chromatography

Notwithstanding the quest of biorelevant analytical methods for better *in vitro* prediction of bioparameters, and the vast amount of research published in the IAM field (see Table 1 for an account of chromatographic systems, experimental data and *in vitro* ~~bioavailability~~ IA parameters), innovative aspects have hardly been ever considered since the launching of the commercial IAM.PC.DD2 and IAM.PC.MG columns based on immobilized PC [36].

209 Usually efforts have been directed to merely apply these commercial columns with
210 standard separation methodologies to the prediction of bioparameters based on
211 chromatographic data vis-à-vis experimental values including the BBB permeability (Log
212 BB), intestine absorption values (P_{eff}), percentage of human oral absorption (%HOA),
213 and *in vitro* absorption or permeability (P_m) using the Madin-Darby kidney (MDCK) cell
214 line [37–51]. Another interesting parameter estimated with commercial IAM
215 chromatographic columns is the octanol/water partition coefficient for non-ionizable
216 compounds ($P_{o/w}$) or for ionizable compounds (D). Also, permeability data estimated
217 from PAMPA can be likewise obtained via IAM chromatographic columns [38,42,44–
218 46,48,50,52]. Although the retention factor (k) obtained from IAM columns is the most
219 common chromatographic variable for estimation of bioparameters, other experimental
220 IAM data such as CHI-IAM (chromatographic hydrophobicity index, [53]) and ΔLog k
221 (residual error of the prediction of Log k using Log P or Log D) are also used. CHI is
222 calculated from the inverse linear relationship obtained by plotting log k versus the
223 acetonitrile concentration in the mobile phase and is defined as the quotient of the
224 intercept (Log k_w, the logarithm of the retention factor with a mobile phase of 100% of
225 water or aqueous buffer) and the slope (the smaller the slope the greater is the reversed-
226 phase type interaction with the IAM sorbent). Some authors use Log k_w instead Log k
227 from IAM to predict bioparameters, although more experimental data is still necessary.
228 In addition, ΔLog k is calculated as the difference between the experimental and the
229 predicted Log k from IAM. For this purpose, the experimental Log k is plotted against
230 Log P or Log D for a set of compounds, and then the predicted log k is obtained from the
231 correlation equation. Usually, the greater the ΔLog k the lower is the log P (or D) because
232 the weaker is the interaction of the given compound with the chromatographic column
233 and thus the prediction is less accurate.

234 In IAM, a mathematical model usually based on partial least-squares is built for a set of
235 compounds utilizing a given experimental parameter from the chromatographic system,
236 i.e., log *k,* CHI or ΔLog *k* but including other molecule properties if necessary (i.e., log
237 *P*, ionization effect, number of polar groups or polar surface area, among others) against
238 *in vivo* data as illustrated in Eq. 1.

239 $$Bp = a + bP^1 + cP^2 + dP^3 \ldots \quad \text{Eq. (1)}$$

240 **Table 1.** Review of published literature using IAM chromatography for prediction of bioparameters and study of interactions with phospholipid membrane
241 published from 2015 up to 2021.

Column[1]	Analytes	Mobile phase Technique	Comparison with	Comments	Reference
IAM PC.DD2 (c, p, pkc) Sphingo-IAM (s, p, pkc)	Drugs	MeOH/DPBS HPLC-UV	Log BB	Preparation of a sphingomyelin-based column to compare with the commercials IAM PC.DD2 and a cholesterol-based column (Cosmosil cholester). Similar predictive performance was obtained for all the columns and not improvement for the combined data.	[37]
Poly(GMA-co-EDMA)@PC (s, m, fs)	Organic acids, lidocaine, and sulfanilamide	MeOH/DPBScH capillary LC-UV	Log %AIRI	Preparation of a Soybean PC column by covalently attachment on monolithic phase thought the phosphate group for capillary LC. The results showed good relationships to predict the bioparameters selected.	[54]
Poly(MDPC-*co*-EDMA) (s, m, fs) IAM PC.DD2 (c, p, pkc)	Proteins and basic drugs	Ammonium acetate buffer/ACN nanoLC and HPLC-UV	Between them	A phosphocholine methacrylate derivative have been synthetized and copolymerized with a crosslinker to obtain a novel monolithic stationary phase. Good correlations with commercial IAM PC.DD2 column was registered.	[55]
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Acidic, basic and zwitterionic drugs	PBS HPLC-UV	Log $P_{o/w}$ Log D Log P_{eff}	The selected commercial columns were used to predict the analytes' Log P_{eff} values showing no relation using the retention factors. However, better results were obtained considering the polar and electrostatic forces.	[38]
IAM PC.DD2 (c, p, pkc)	Neutral, acidic, basic and zwitterionic drugs	PBS/ACN HPLC-UV	%HOA Log D MDK Cell	Retention indices on the commercial column were used to predict different parameters of 22 drugs, including	[44]

			lines Log P_{eff}	the human oral absorption. The results showed a limited prediction ability.	
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	~~drugs~~Drugs	PBS/ACN HPLC-UV	Log BB PAMPA-BBB Log $P_{o/w}$	Δlog k_w^{IAM} was used to predict the BBB passage and the present study demonstrates the soundness of this parameter to predict it. In addition, it showed superior capacity than PAMPA-BBB and Log $P_{o/w}$.	[45]
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Drugs	PBS HPLC-UV	Log P_{eff} Log $P_{o/w}$ Log D	Δlog k_w^{IAM} were used to predict the intestinal absorption of drugs with good results. Also, the authors interpret that polar/electrostatic forces between drugs and phospholipids play a major role in the passage through biomembranes.	[46]
IAM PC.DD2 (c, p, pkc) Poly(MDPC-*co*-EDMA) (s, m, fs) Poly(MSDPC-*co*-EDMA) (s, m, fs)	Proteins Pharmaceutical compounds	H_2O/ACN (both with 0.1% TFA) nanoLC and HPLC-UV	Between them	MCP based on phosphocholine and MDSPC based on 11-aminoundecanoic acid a phosphocholine derivative were used to act as methacrylate monomers in the preparation of monolith stationary phases. Both synthetized columns were compared with the commercial IAM column showing good correlations.	[56]
Regis IAM Fast Mini Screening (c, p, pkc)	Drugs	AAB/MeOH HPLC-UV and TOF-MS	Log BB	The commercial IAM column was used in combination with MS to predict the BBB passage obtaining solid statistics. Although, the common DPBS solvent was substituted by an AAB buffer, the predictive power was similar.	[47]

IAM PC.DD2 (c, p, pkc)	Drugs Organic compounds	AAB/ACN HPLC-UV	PAMPA MDCK cell lines	253 molecules, including few organic compounds apart from drugs were used to study the IAM commercial column to predict passive permeability obtained by PAMPA and MDCK systems. The combination of IAM data with polar surface area led to satisfactory predictions.	[48]1
IAM PC.MG (c, p, pkc)	Bisphenols	PBS/CAN HPLC-UV	Log BB Skin and Corneal permeability Cell toxicity	IAM commercial column was used to stablish relationships between *in vitro* toxic activity of bisphenols and phospholipophility obtained by retention on IAM column. The results showed good correlations where more interaction with the phospholipid means more toxicity.	[49]
IAM PC.DD2 (c, p, pkc)	Penetrating and no-penetrating BB compounds	PBS/ACN HPLC-UV	Log $P_{o/w}$ P_m Plasma protein binding Log BB	The manuscript is focused on the use of IAM retention factors, PPB and permeability to predict the BBB. The results showed that more than one parameter is necessary to obtain reasonable predictions.	[50]
IAM PC.DD2 (c, p, pkc)	Perfluorinated alkylated substances	AAB/ACN UPLC-MS/MS	Cellular accumulation	Phospholipophility obtained by retention factor on the IAM commercial column was used to predict the cellular accumulation in different cell types showing high correlations.	[51]
IAM PC.DD2 (c, p, pkc)	Peptides	AAB/ACN HPLC-UV	Log BB	The chromatographic data was used to derive estimated *in vivo* distribution, drug efficiency, brain tissue binding, fraction unbound in brain and plasma, brain to plasma ratio and cell partition.	[39]

IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Flavonoids	H_2O/ACN HPLC-UV	Cell-based permeability	IAM stationary phases were used to obtain correlations between cell permeability literature data. Both stationary phases showed comparable performance towards Caco-2 cell permeability.	[40]
IAM PC.DD2 (c, p, pkc)	~~Psychopharmacs~~Psychopharmaca	PBS/ACN HPLC-UV	Log BB	Gradient reverse elution was used to develop a linear correlation between IAM column retentions and Log BB showing extremely good results for eleven drugs.	[41]
IAM PC-MG (c, p, pkc)	Pesticides	PBS/CAN HPLC-UV	LC_{50} LD_{50}	The potential of IAM to predict ecotoxicological endpoints of 39 pesticides was evaluated. IAM retention factors showed promising predictions respect to the ecotoxicological risk	[57]
Bovine brain PS liposomes (s, fs, fs)	Drugs	40 mM HEPES CEC-UV	Log BB PAMPA-BBB	A novel *in vitro* method based on the use of liposomes in capillary electrochromatography was used to predict *in vivo* data Log BB and cell permeability.	[42]
IAM PC.DD2 (c, p, pkc)	Beyond rule of 5 molecules	AAB/ACN UV	P_m	Study of lipophilicity using an IAM column for beyond rule of 5 molecules. In addition, the obtained results were used to check the relationship with solid permeability.	[43]

[1] s: synthetized; c: commercial; s/c: synthetized based on a commercial column; m: monolith; p: particles; fs: fused silica; pkc: packed column

Abbreviations: Glycidyl methacrylate (GMA); Ethylene glycol dimethacrylate (EDMA); Phosphatidyl choline (PC); Methanol (MeOH); AAB: Ammonium acetate buffer; Dulbecco's Phosphate buffer saline (DPBS); Acetonitrile (ACN); 12-methacryloyl dodecylphosphocholine (MDPC); Chromatographic hydrophobicity index (CHI); Jejenum absorption values (Log P_{eff}) 1-dodecanoyl-2-(11-methacrylamidoundecanoyl)-sn-glycero-3-phosphocholine (MDSPC); Thrombin (THR); Time of flight mass spectrometry (TOF-MS); Ammonium bicarbonate buffer (ABB); human oral absorption (%HOA); Madin-Darby canine kidney (MDCK); Parallel artificial membrane permeability assays (PAMPA); Blood-brain-barrier (BBB); Sorption affinity into a phospholipid membrane (K_{PLIPW}); Plasma protein binding (PPB); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); 1,2-diacyl-sn-glycero-3-phospho-l-serine (PS).

In this equation, the *in vivo* bioparameter (*Bp*) is explained by a constant (*a*) and a series of parameters (P^n, where *n* is the number of the parameter) multiplied by its slope (*b, c, d*, etc.). Once built, the model is applied to target analytes for estimation of the *in vitro* ~~bioavailability~~ IA parameter ($Bp_{estimated}$) of every analyte. Good correlations between $Bp_{estimated}$ and *in vivo* BP are then sought for validation and acceptance of the model. Practically all the papers in the literature focused on corroborating the utility of IAM columns for pharmaceutical drugs and drug development [43], with correlations (R^2) of Log *k* from IAM against *in vivo/ex vivo* parameters using real biological membranes usually ranging between 0.7 and 0.8. Novel bioparameters that are proven to be appropriately estimated *in vitro* by IAM include cellular accumulation (predicted by CHI-IAM) [51], cell toxicity using Log *k* [49] and ecotoxicological risks using a model, which includes log *k* from IAM chromatographic data and other physicochemical and molecular descriptors such as hydrogen bond donor/acceptor properties, among others [57]. In the case of ΔLog *k,* an inverse relationship (negative slope) against with the selected bioparameter is sought because high ΔLog *k* (i.e., high residuals) usually stands for a weak interaction of the compound with the biomimetic system (and expected with real membranes) while low ΔLog *k* (low prediction error) is obtained for compounds with a high-sorbent interplay, and thus with potential high ~~bioavailability~~ IA~~and absorbability~~.

It should be noted that compounds other than drugs have been scarcely studied by IAM, yet some environmental pollutants such as alkylbenzenes, polycyclic aromatic hydrocarbons (PAHs), bisphenols and perfluorinated alkylated substances (PFAS) have been targeted to [49,51,58]. In our opinion, the use of bioinspired stationary phases to estimate cellular accumulation PFAS is a promising approach in human exposomic studies [51]. Literature results showed that the cellular accumulation is highly dependent upon lipid binding expressed as CHI-IAM at pH 7.4 [59]. If other parameters, such as

Log *D* at pH 7.4 and Log *P* are added to CHI obtained by IAM, according to Eq. 1, the predictive results are greatly improved.

Trends in the IAM field are focused on the synthesis and testing of novel stationary phases with a variety anchored phospholipids [37,58] or the fabrication of methacrylate-based chromatographic monoliths for microscale separation by using chemically modified phospholipids with vinyl moieties to undergo UV/thermal copolymerization (Fig. 3) [54–56].

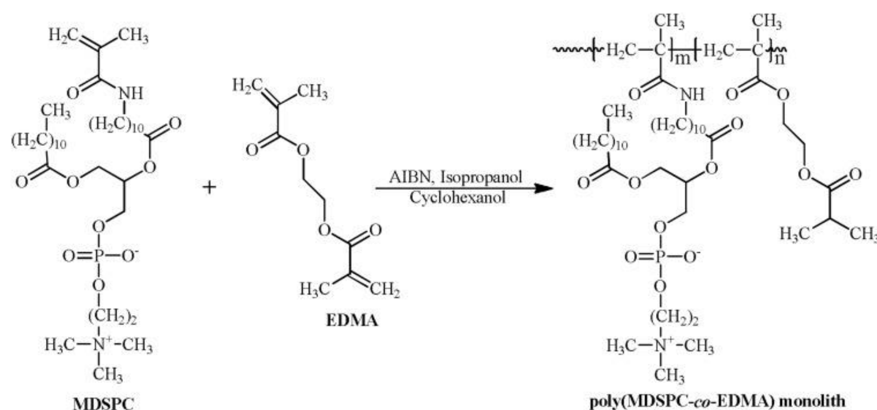


Fig. 3. Schematic diagram of the preparation of a polymer containing a modified phospholipid with vinyl groups. MDSPC: 1-dodecanoyl-2-(11-methacrylamidoundecanoyl)-sn-glycero-3-phosphocholine and EDMA: ethyleneglycoldimethacrylate. Reproduced with permission of Elsevier [56].

Special attention deserves the IAM monolith column proposed by Moravcovà *et al.* [54]. In this case, the PC was uniquely anchored through the phosphate group to the column surface, thus changing the common biomolecule orientation in IAM columns, for which the alkyl chains are usually bounded to the column surface. Similar correlations against *in vivo* parameters than those reported for with commercial columns were obtained for the studied analytes (dye, amines, anti-inflammatory, antibacterial, antifungal, analgesics,

292 and bronchodilator drugs), although no direct comparison with commercial columns was
293 performed. However, the chemical procedure for binding of PC through the polar
294 moieties seems not straightforward as compared to the facile fabrication protocols of
295 commercial IAM.PC.DD2 and IAM.PC.MG columns. Table 1 shows that phospholipid
296 monomers have been employed in all IAM dynamic approaches to generate a planar lipid
297 monolayer onto material surfaces in the attempt to simulate membrane interactions with
298 xenobiotics. Nevertheless, membranes of eukaryotic cells are constituted by phospholipid
299 bilayers in a spherical/ellipsoidal shape, which are far from being mimicked with the
300 monolayers used to date. To tackle this issue, Godyń *et al.* [42] proposed an elegant
301 solution by coating silica capillaries with 1-palmitoyl-2-oleoyl-sn-glycero-3-
302 phosphocholine (POPC) and 1,2-diacyl-sn-glycero-3-phospho-l-serine (PS)-based
303 liposomes as lipid membrane surrogates to allow for electrostatic and Van der Waals
304 interactions with the target species. However, the experimental correlations with IAM
305 data (Log *k*) vs Log *BB* and Log P_e (*in vivo*) were quite poor (R^2 = 0.426 and 0.374,
306 respectively).

307 In the design of IAM methods, researchers need to incorporate runs within a certain range
308 of pH according to the pH gradient of the gastrointestinal tract (2.0-8.0) to account for
309 potential variations across the retention factor of the target compounds [44,60]. In
310 addition, a common practice is to analyse only one analyte at a time by HPLC with
311 UV/Vis detection under isocratic conditions employing low % of organic phase. Because
312 the separation is performed under non-ideal chromatographic conditions, but appropriate
313 to trigger membrane interactions, poor peak resolution is commonly observed, and thus
314 multicomponent analysis are usually not feasible. In this sense, the use of mass
315 spectrometry detection offers the opportunity to detect several compounds
316 simultaneously. Nevertheless, attention should be paid to the HPLC conditions to avoid

317 incompatible buffers with mass spectrometry, such as PBS, and select compatible eluents
318 that could potentially mimic physiological conditions.

319 **2.2.2 Biopartitioning micellar chromatography**

320 Biopartitioning micellar chromatography (BMC) was proposed by Escuder-Gilabert *et*
321 *al.* in 2004 [61]. BMC can be described as a chromatographic method in which the mobile
322 phase is composed by a surfactant system (commonly Brij35, non-ionic surfactant with
323 hydroxyl moieties) over its critical micellar concentration, with the surplus of monomers
324 being absorbed adsorbed onto the C18/C8 bed of a reversed-phase column to create a
325 C18/C8-surfactant bilayer. The double equilibria generated between the compound(s) and
326 the micelles (acting as pseudostationary phases) and the stationary phase surface bilayer
327 (surfactant +C18/C8 chains) that mimics both the polar and hydrophobic regions of the
328 lipid membranes are expected to simulate closely those interactions occurring in *in vivo*
329 oral absorption of drugs, BBB penetration or intestinal absorption permeability, among
330 other processes [62]. However, BMC, apart from poor column efficiency and the weak
331 solvent strength of micellar eluents, only capitalizes upon passive diffusion and therefore
332 if other underlying mechanisms are involved (*e.g.* via paracellular route or active
333 transport), large deviations can be observed,. Therefore, BMC, in some instances, only
334 can provide a limited insight into the actual drug absorption in humans and biota. A
335 selection of the most interesting publications and estimated bioparameters using BMC
336 within the time span of 2015-mid 2021 are summarized in Table 2.

337 C18 reversed-phase columns have been the common choice of stationary phases [62–64]
338 because the C18 chains foster interactions by Van der Waals forces with the alkyl chains
339 of the surfactant monomers generating the bespoke surface bilayer. However, some
340 authors recommended alternative stationary phases, such as cyanopropyl [65] or

341 aminopropyl [66] attempting to obtain more polar surfaces for low-retained analytes in
342 C18 columns. For example, De Vrieze *et al.* [67] combined a classical C18 column with
343 synthetized miltefosine which was used as a surfactant in BMC for a better mimicry of
344 biological membranes than those obtained by other surfactant counterparts to predict HIA
345 and log BB values. In that work, for example, PLS was used to predict Log BB using the
346 Log *k* obtained from BMC and other molecular descriptors as shown in Eq. (2)

347 $$\text{Log BB} = -2.669 + 0.234 \times \text{Log } k + 0.699 \times \alpha - 0.048 \times \text{P} - 0.002 \times \text{WS}_{7.4} + 0.009 \times \text{PB}$$
348 $$+ 0.034 \times \text{HIA} - 0.017 \times \text{PSA} + 0.167 \times \text{HBA} \quad \text{Eq. (2)}$$

349

350 **Table 2.** Review of published literature using BMC technique for prediction of bioparameters published from 2015 up to 2021.

Column[1]	Analytes	Mobile phase Technique	Comparison with	Comments	Reference
C18 (s/c, p, pkc)	Drugs	Brij-35 in PBS HPLC-UV	LC50	A two-dimensional liquid chromatography method was developed using a BMC separation in first dimension and C18 reversed phase in the second dimension to study the identification, bioactivity and toxicity of drugs with a time-saving and low-cost system. The second dimension improve the weak separation ability of BMC.	[63]
C18 (c, p, pkc)	Drugs	Miltefosine aqueous solution TOF-MS	Log BB HIA	A synthesized surfactant (miltefosine) that mimics better the biological layers has been used for BMC. The retention factors in combination with other descriptors were used to develop models to predict Log BB and HIA and the correlation coefficients were between 0.37 and 0.88.	[67]
C8 (c, p, pkc)	Drugs	PC and SDS HPLC-UV	Log D	The use of microemulsions in presence of mixed micelles and oil has been used to mimic the biomembrane. The system showed better correlations for log *D* than other IAM chromatograpy. However, the authors did not use the system to predict other bioparameters.	[68]
Cyanopropyl column (c, p, pkc)	Drugs	NaDC aqueous solution HPLC-UV	HIA	Alternative BMC system using bile salts have been used to predict intestine permeability expressed as HIA for pharmaceutical compounds obtaining r^2 between 0.75 and 0.86.	[65]
C18 (s/c, p, pkc)	Structurally unrelated analytes	SDS aqueous solution HPLC-UV	Log BB	Partial least square method was used to predict BBB using retention factors of BMC and other topological and physicochemical parameters. The results showed high correlations ($r^2 = 0.83$). Also, when IAM columns were used ($r^2 = 0.78$).	[64]

Zorbax Extend-C18 (c, p, pkc)	IRs/α-Ars, drugs	Brij-35 in PBS HPLC-UV	Log BB	BMC retention factors were used to estimate the BBB permeability of different drugs. The correlations of BMC showed higher correlation factors ($r^2 = 0.77$) than common reversed-phase ($r^2 = 0.58$).	[62]
APS (c, p, pkc)	Drugs	NaDC aqueous solution HPLC-UV	HIA	In this study the prediction of HIA was extended to more compounds thanks to the incorporation of an aminopropyl column. The micelle-water partition coefficients were calculated and combined with other descriptors and used to predict HIA showing correlations (r^2) in the range 0.72-0.85.	[66]
- (c/s, -, fs)	Drugs	Brij35, Tris and HEPES HPLC-UV	Log BB	Biopartitioning micellar electrokinetic chromatography (BMEKC) as alternative to common BMC was used to estimate the BBB of drug candidates. The proposed methodology showed similar correlation coefficients ($r^2 = 0.73$) compared to that found on conventional BMC ($r^2 = 0.75$)	[69]

351 [1] s: synthetized; c: commercial; s/c: synthetized based on a commercial column; m: monolith; p: particles; fs: fused silica; pk c: packed column

352 Abbreviations: Human intestinal absorption (HIA), sodium deoxycholate (NaDC), β-hydroxy-β-arylalkanoic acids (HAA), Quantitative Structure-Retention Relationship
353 (QSRR), imidazoline/α-adrenergic receptor (IRs/α-ARs), anionic sodium dodecyl sulfate (SDS), reversed- phase aminopropyl column (APS)

354

355 in which α is the total molar charge at pH 7.4, P is the polarizability, WS7.4 is the aqueous
356 solubility at pH 7.4, PB is the plasma protein binding, HIA is the human intestinal
357 absorption, PSA is the polar surface area and HBA is the hydrogen bond acceptor capacity
358 for every target compound. The authors investigated 36 drugs, and good correlation
359 coefficients for predicted log BB against *in vivo* log BB were obtained ($R^2 > 0.72$), yet
360 not only chromatographic and physicochemical parameters from the literature but *in vivo*
361 data such as HIA were needed to build a reliable model. The relevance of this work is the
362 usage of a phosphocholine-based surfactant as a model of cell membrane because
363 phospholipids cannot be used as reliable surfactants because of solubility issues.
364 However, as can be seen by the reported results, the intricate interactions in live
365 organisms cannot be explained merely by the underlying passive diffusion in BMC, even
366 with phosphocholine-based surfactants. Other authors proposed hybrid micelles of PC
367 and sodium dodecyl sulfate (SDS) in the presence of an organic phase (mixture of *n*-
368 butanol and ethyl acetate) that are aimed at predicting biomimetic parameters by
369 microemulsion liquid chromatography (MELC) [68]. The three-phase
370 (microemulsion/water/column) model (Fig. 4) features better prediction of Log *D* than
371 that obtained by IAM chromatography. Notwithstanding the fact that the authors
372 suggested that permeability descriptors can be appropriately described with MELC
373 (determined by principal components analysis), potential correlations between *in vivo* and
374 MELC data were regrettably not investigated.

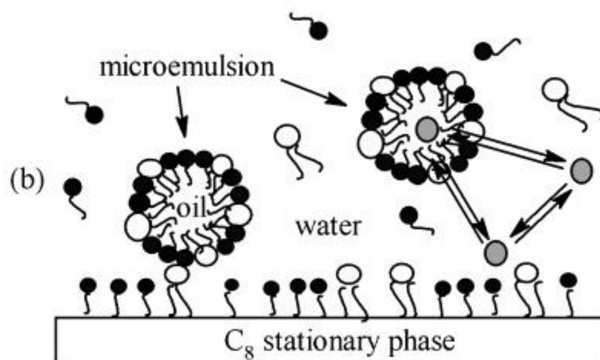


Fig. 4. Schematic representation of proposed MELC interphase using C8 stationary phase and a microemulsion constituted by PC (white circles), SDS (black circles) and an oil. The target compound is represented in grey color. Reproduced with permission of Elsevier [68].

In another work, Waters *et al.* [65] exploited sodium deoxycholate (bile salt) and the reversed phase cyanopropyl column to predict HIA for various drugs (R^2 = 0.75-0.86). In the same way, Shokry *et al.* [66] selected the same bile salt in combination with an aminopropyl column to study a larger number of compounds with contrasting behaviors related to the affinity to the micelle based on hydrophilic/lipophilic interactions. The micelle-water partition coefficients were calculated and showed that antibinding compounds (to the micelle) have better retention onto the stationary phase with the increase of surfactant concentration while non-binding compounds do not show alteration of their retention times with changes in micelle concentration. The partition coefficients, in combination with other descriptors such as molar volume and aqueous solubility, were used to predict HIA with relatively good results against *in vivo* HIA (R^2 = 0.72-0.85). However, the two columns described in this paragraph showed similar correlations against *in vivo* HIA for an alike pool of drugs thus demonstrating that both micelle

systems in combination with reversed-phase stationary phases bearing polar moieties are biorelevant.

In our opinion, BMC has made tremendous strides recently to leverage its main advantages: (i) simultaneous simulation of a number of pharmacokinetic parameters with a single measurement, (ii) data robustness, (iii) low cost, (iv) green credentials, and (v) flexibility in the selection of the stationary phase and surfactants. However, gradient conditions are herein excluded, with the consequent increase of the analysis time under isocratic conditions [70]. BMC~~and its~~ fundamentals have been also incorporated in other separation techniques, such as micellar electrokinetic chromatography (MEKC). In this electroseparation technique, the pseudo-stationary phase added to the background electrolyte consists of an aqueous surfactant solution. In fact, ~~MECK~~ MEKC and BMC can be synergistically combined in the so-called biomimetic ~~MECK~~ MEKC (~~BMECK~~BMEKC) in which the electrophoretic conditions simulate the interaction between analytes and biological membranes [69]. The BMEKC system proposed by Ciura *et al.* [69] provides a similar correlation coefficient than that reported by BMC methods for *in vitro* BBB evaluation (R^2 = 0.73 against *in vivo* BBB) but with significantly lower consumption of reagents thus coping with green analytical chemistry principles.

2.2.3 ~~Biomimetic liquid chromatography~~Immobilized plasma protein chromatography

~~BLC~~ IPPC is a chromatographic technique capitalizing upon the measurement of protein binding throughout the cell membrane or the bloodstream using stationary phases modified with proteins such as human serum albumin (HSA) or alpha-1-acid glycoprotein (AGP) [5] among others. The interaction of the target compounds with proteins is an

important bioparameter inasmuch as their pharmacodynamic behaviors, cell permeation and drug-drug interactions might be significantly altered. Up to the date, CHIRALPACK HSA and its counterpart of AGP have been the common stationary phases to measure the protein binding for different analytes such as drugs [39,71], BBB and non-BBB penetrating compounds [50], phytoestrogens [72] and bisphenol analogues [73]. In these cases, the majority of the works reported binding values or their comparison with Log *P*. However, Valko *et al.* [71] elegantly attempted to estimate the steady state volume of distribution (V_{dss}) of target drugs, which is a key parameter for setting drug doses, by using log *k* from HSA column (related to the binding protein value) and also from IAM column (membrane permeation) to predict the *in vivo* V_{dss}, although low correlations were observed between *in vivo* and chromatographic data (R^2 = 0.56-0.66). Therefore, some authors proposed new column materials to measure protein binding values *in vitro*. As an example, Ma *et al.* [74] fabricated a frontal silica-based affinity chromatographic column with hierarchical mesopores and penetrable macropores containing covalently attached BSA by Schiff base. The columns enabled the enantioseparation of D/L tryptophan and the frontal affinity chromatographic analysis of imatinib mesylate and demonstrated that there is a single type of binding between the analyte(s) and BSA. The authors signaled that these columns will open new avenues for the measurement of the protein-drug interaction of low-to-moderate retained analytes in other chromatographic materials. In another interesting example, Liang *et. al.* [75] immobilized angiotensin II type I receptor (AT1R) to probe antihypertensive compounds. For this purpose, the AT1R was expressed using *E. coli* and after cell lysate, the protein was immobilized onto 6-chlorocaproic acid-activated amino polystyrene microspheres. The as-prepared columns were used for elucidating drug-receptor binding kinetics and thermodynamic parameters. The authors expanded the use of the AT1R columns for the identification of puerarin and

rosmarinic acid as antihypertensive compounds in natural products although both species are far away from the requirements of a drug candidate. In short, IPCC has been mostly linked to the use of the common HSA and AGP columns, which are readily available. However, it is necessary to prepare novel phases that offer better interaction profiles to obtain a deeper understanding of the involved processes in biological systems. In fact, there is a quest of novel IPCC columns containing other proteins, such as globulins, phosphoproteins, lipoproteins and/or C-reactive protein to account for a broad range of analyte-protein interactions. In addition, the synergetic combination of various proteins in a single material may contribute to unveil the underlying mechanisms in IA processes.

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2.2.4 Miscellaneous biomimetic systems

Apart from standard biomimetic structures encompassing PC, proteins, and surfactant micelles as artificial membranes, other biorelevant systems have been used to elucidate the complex interactions occurring between the target compounds and the lipid membrane. For example, Stephen *et al.* [76] harnessed cellular membrane affinity chromatography (CMAC) which consists of the immobilization of cell membrane fragments with fully functional transmembrane proteins onto IAM stationary phases. The early usage of these stationary phases was limited to the binding characteristics of immobilized transmembrane proteins. However, as demonstrated in that work, CMAC can be expanded to the identification of pharmacologically active metabolites from natural products. In any case, CMAC columns do not serve as universal tool in drug discovery but do speed up the process of target identification. The bioanalytical and pharmacological potential of these columns is still in its infancy, but we do expect the upward trend of CMAC in ~~BLC~~ IPPC and IAM to continue in the foreseeable future.

465 Notwithstanding the fact that liquid chromatography has been by far the separation
466 technique of choice for resembling biological processes *in vitro*, other dynamic
467 techniques such as supercritical fluid chromatography (SFC) [77] and thin-layer
468 chromatography (TLC) [78] have been also adopted to predict bioparameters, namely,
469 permeation through BBB. For example, Russo *et al.* [77] used SFC to estimate the PSA
470 of target compounds, and in combination with other parameters (viz, IAM retention
471 factor, water accessible surface and number of aliphatic carboxylic acids and
472 phenol/enol/carboxyl/hydroxy groups, see eq. 1) succeeded in predicting Log BB of
473 sixty-nine acid, base, neutral and amphoteric substances with good correlations with *in*
474 *vivo* Log BB data (R^2 = 0.81). Log BB can be also predicted using the chromatographic
475 data (Rf) obtained from a reversed-phase C18 TLC separation [78]. In addition, the
476 combination of Rf with PSA was suggested as a universal predictor of brain absorption
477 on the basis of excellent correlations with *in vivo* BB data (R^2 = 0.9). In summary,
478 different separation systems, including SFC, TLC and the aforementioned BMECK [69]
479 can be harnessed to the prediction of Log BB, which thus is not exclusively dependent on
480 *in vitro* data by high performance liquid chromatography.

481 **3. Conclusion and outlook**

482 In this review, the state-of-the-art of artificial membranes in (bio)analytical applications
483 has been critically dissected. The research developments since 2015 up to mid-2021 in
484 terms of material science have been rather limited and the majority of the publications
485 continue employing standard/customary methodologies (*e.g.* PVDF coated supports) or
486 commercial systems (e.g. IAM.PC.DD2 column). In the case of static artificial
487 membranes, trends are focused on the combination of mucus layers with PVPA systems
488 [22] and gastrointestinal fluids that led to high correlations with *in vivo* data. On the other
489 hand, some innovative methods have been reported in dynamic modes (IAM

chromatography, BMC and ~~BLC~~IPPC) aimed at ameliorating ~~bioavailability~~ IA results. For example, novel materials for IAM chromatography have been prepared by surface attached phospholipids [54]. The idea behind is to improve the *in vivo/in vitro* correlations of bioavailability values obtained with commercial columns ~~with usually $R^2 < 0.85$.~~ The incorporation of novel choline-based surfactants [67] and dedicated surfactants such as bile salts [65] have been the most interesting trends in BMC to improve ~~bioavailability~~ IA predictions. Nevertheless, the passive diffusion through the lipid membrane mimicked by AIM and BMC is not sufficient to simulate the intricate interactions occurring in cell membranes during the absorption of compounds. For this reason, ~~BLC~~ IPPC can be used to simulate other membrane-target interactions such as protein binding using columns with immobilized AGP. However, other proteins such as AT1PR has been attached to the stationary phase [75] which demonstrates the fact that ~~BLC~~ IPPC is not only limited to standard membrane/serum proteins but other specific interactions with other proteins and biomolecules can be explored.

To shed light into the complex phenomena of ~~bioavailability~~IA, interest has grown on alternative techniques such as CMAC which uses cell membrane fragments [76], BMECK [69], MELC [68], SFC [77] and TLC [78] to leverage the possibilities offered by liquid chromatographic methods. To the best of our knowledge, most of the studies dealing with artificial membranes focused on the absorption of pharmaceutical compounds, yet the ~~bioavailability~~ IA of legacy and emerging contaminants has been scarcely studied.

Our vision is that the development of hybrid/smart materials involving monoliths, nanomaterials, metal organic frameworks and/or 3D printed templates in combination with biomolecules or membrane surrogates is expected to open new avenues for

514 mimicking the human absorption/~~bioavailability~~ IA of targets on account of the plethora
515 of interaction mechanisms available that resemble those of the eukaryote cell membranes.

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Figure Captions

Fig. 1. Scheme of the different pathways for endogenous and xenobiotic compounds to pass through the lipid membrane. Passive diffusion (A); facilitated diffusion (B); active transport (C); endocytosis-facilitated process (D). Created with BioRender.com.

Fig. 2. Scheme of the PAMPA procedure and magnification of the passive diffusion of targets through lipid bilayers. Created with BioRender.com.

847 **Fig. 3.** Schematic diagram of the preparation of a polymer containing a modified
848 phospholipid with vinyl groups. MDSPC: 1-dodecanoyl-2-(11-
849 methacrylamidoundecanoyl)-sn-glycero-3-phosphocholine and EDMA:
850 ethyleneglycoldimethacrylate. Reproduced with permission of Elsevier [56].

851 **Fig. 4.** Schematic representation of proposed MELC interphase using C8 stationary phase
852 and a microemulsion constituted by PC (white circles), SDS (black circles) and an oil.
853 The target compound is represented in grey color. Reproduced with permission of
854 Elsevier [68].

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1 # Human artificial membranes in (bio)analytical science:
2 # Potential for *in vitro* prediction of intestinal absorption-A
3 # review

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24 **Keywords:** Artificial membranes; biomimetic; parallel artificial membranes;
25 immobilized artificial membrane chromatography; biopartitioning micellar
26 chromaotography; biomimetic liquid chromatography; intestinal absorption.

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Abstract

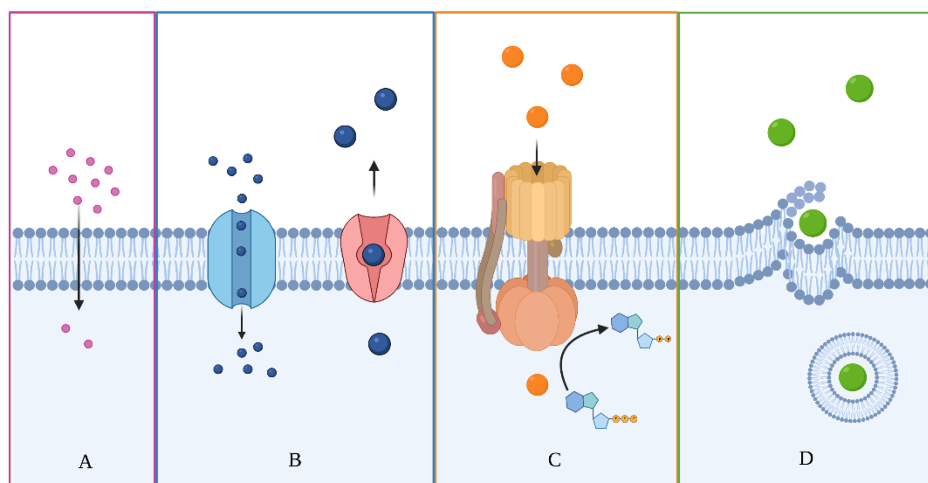
Artificial membranes for investigation of the human absorption (oral, dermal or respiratory) of target organic compounds are aimed at mimicking the interactions occurring within the lipid membrane. Biomolecules such as proteins are also integral components of the lipid membranes and play a pivotal role towards understanding the complex mechanisms of human absorption. In this review, we will differentiate biomimetic platforms based on static (batchwise) and dynamic modes. In the former, a synthetic membrane placed between two phases (donor and acceptor) mimics a given biological system to study permeability. Parallel artificial membrane permeation assays are the most common approaches for static mode. As to dynamic modes, there is a plethora of bioanalytical techniques such as immobilized artificial membrane chromatography, biopartitioning micellar chromatography or immobilized plasma protein chromatography. In any case, all of the dynamic approaches capitalize upon analytical separation techniques such as liquid chromatography and the use of the chromatographic factors to predict permeability and other bioparameters. However, improvements in the fabrication of novel sorptive materials or the development of innovative techniques/approaches to enhance the prediction capability of permeability by simulated membranes has been left in the background. For this reason, this review covers the current state-of-the-art of immobilized artificial membranes in bioanalytical science with particular focus on new materials and techniques reported from 2015 to mid-2021. Future perspectives related to the fabrication of innovative artificial membranes for *in vitro* intestinal absorption studies have been highlighted so as to encourage fundamental studies in this research area.

1. Introduction

Human absorption refers to a pharmacokinetic process on the basis of which a given amount of a target compound is able to pass from external sources (oral, dermal or respiratory) through cell membranes and, therefore, enter into a living organism [1,2]. For accurate assessment of the human absorption, the variety of potential interactions between the target species and the cell plasmatic membrane including dipole-dipole, hydrogen bond donor/acceptor, London, cation-π and electrostatic interactions need to be thoroughly studied, yet this is a very complex process that is dominated by the occurrence of different biomolecules: lipids, proteins, and polysaccharides, among others [3]. In addition, the knowledge of the absorption conditions (pH, temperature, fluid composition, etc.) is necessary because might affect the lipophilic nature of the target compound. In this sense, insight into the human compartment from which the target compound is going to be absorbed is particularly relevant because, for example, the pH in the gastric fluid (1.0-1.4) differs substantially from that of the plasma (*ca.* 7.4) or that of the small intestine (6.5-8.5) [3] and thus the intestinal absorption (IA) of ionizable compounds might be significantly altered.

The pathways for compounds (drugs, nutrients, unwanted xenobiotics, etc.) to pass through the lipidic membrane are severalfold and are deeply discussed in previous reviews [3,4] as summarized in Fig. 1. Briefly, the absorption processes could be divided in: (i) passive diffusion in which a net movement of the compound from one side of the membrane to the other is related to the concentration gradient (Fick's law) (Fig. 1A). The partition coefficient (P) in octanol/water system is the most common parameter to express the lipophilicity of a chemical, and therefore the ability to be transported by diffusive transport; (ii) protein-mediated transfer that uses membrane proteins as carriers to generate pathways through the lipid membrane (facilitated diffusion, see Fig. 1B); (iii)

77 active transport that allows the movement of molecules against the concentration
78 gradient, polar repulsion, or other resistive forces using membrane proteins and
79 employing energy (adenosine triphosphate, ATP) (see Fig. 1C); (iv) endocytosis-
80 facilitated process that consists of the transport of large molecules (proteins,
81 polysaccharides, etc.) by engulfment of the compound by the cell membrane itself (see
82 Fig. 1D).



83

84 **Fig. 1.** Scheme of the different pathways for endogenous and xenobiotic compounds to
85 pass through the lipid membrane. Passive diffusion (A); facilitated diffusion (B); active
86 transport (C); endocytosis-facilitated process (D). Created with BioRender.com.

87 Up to date, a vast amount of the literature is focused on investigating the interactions
88 between drugs and the lipid membrane and also with membrane proteins in order to
89 elucidate different biologically-relevant parameters, such as Log $P_{oct/water}$ (in neutral, P^N,
90 or ionized, D), Log BB (blood-brain), Log P_{eff} (effective intestinal/Jejeunal permeability)
91 or protein binding, among others, as summarized in recent review articles [3,5,6]. Log
92 BB is an important parameter that is defined as the logarithm of the ratio of the
93 concentrations of a target compound in the brain and in the blood under equilibrium
94 conditions. This bioparameter gives insight into the blood-brain barrier (BBB)

95 permeability. For *in vivo* measurements, the concentration of the target compound is
96 analyzed in the brain and blood of a rat previously administrated with the compound [7].
97 The Log P_{eff} is the logarithm of the *in vivo* human effective permeability of the target
98 compound in a specific zone of the intestine (duodenum, jejunum or ileum) and can be
99 calculated by measuring the permeation rate of the target compound during intestinal
100 perfusion [8]. Although the *in vivo* approaches are the most accurate methods to predict
101 bioparameters, the use of *in vitro* cell-free methodologies exploiting QSAR (quantitative
102 structure-activity relationship) calculations have attracted the interest of researchers over
103 the last few years. To this end, artificial biomimetic membranes (ABM) using cell-free
104 permeation systems [9] in batchwise mode, and immobilized artificial membrane (IAM)
105 chromatography, biopartitioning micellar chromatography (BMC) and immobilized
106 plasma protein chromatography (IPPC) in dynamic mode have emerged as appealing *in*
107 *vitro* counterparts. With respect to ABM methods, the parallel artificial membrane
108 permeability assay (PAMPA) is commonly reported in the literature, although other
109 alternatives, such as the phospholipid vesicle-based permeation assay (PVPA), and the
110 Permeapad® and the artificial membrane insert (AMI) systems are worth mentioning
111 [10]. In the original PAMPA, egg lecithin containing a mixture of phospholipids
112 (phosphatidyl choline, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI) as
113 major cell membrane components, dissolved in n-dodecane, is employed to mimic the
114 lipid membrane of eukaryote cells [11]. For this purpose, a polyvinylidene fluoride
115 (PVDF) filter is soaked in the lipid solution and placed between two liquids, the donor
116 phase and the acceptor phase until reaching steady state. However, this ABM method
117 underestimates the fraction of target species absorbed due to the absence of other key
118 interactions occurring in biological systems. Therefore, dynamic variants that are focused
119 on separation techniques, mainly chromatography, namely, IAM, BMC and IPPC are

120 gaining momentum [11]. In short, lipid monolayers based on phospholipids are in IAM
121 chromatography covalently linked to silica or monolithic stationary phases. The retention
122 factors of target compounds using IAM columns in liquid chromatography are related to
123 bioparameters [3]. In BMC, micellar pseudostationary phases mimicking the liposome
124 structure are adopted [12]. IPPC measures the binding of target species with proteins in
125 the blood stream or membrane surfaces using stationary phases containing immobilized
126 human serum albumin (HSA) or alpha-1-acid glycoprotein (AGP) [5], respectively.

127 This review is aimed at critically assessing *in vitro* chromatographic and static methods
128 mimicking biological membranes (lipid bilayers) that have been recently resorted to the
129 prediction of bioparameters, with emphasis on innovations of chromatographic materials
130 and biorelevant cell surrogates, and their possibilities to act as predictors of the IA of
131 drugs and pollutants.

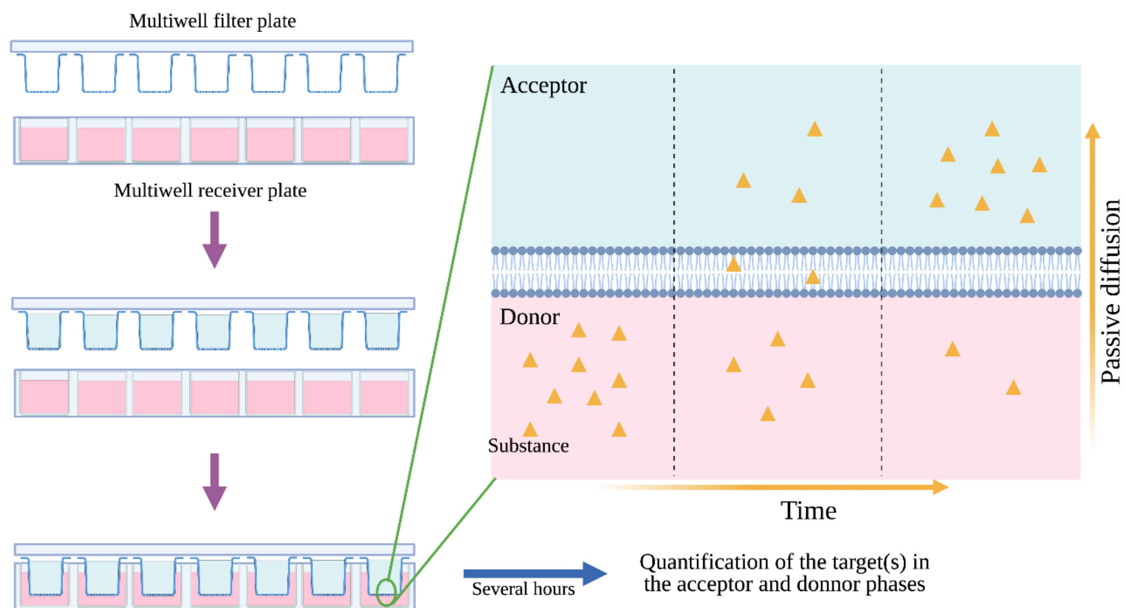
132

133 **2. Cell-free artificial membranes for permeability studies**

134 **2.1. Static (batchwise) systems: Artificial biomimetic membranes**

135 As indicated above, PAMPA is the most common ABM cell-free methodology to explore
136 the *in vitro* permeability/IA of drugs and contaminants in the human organism. A scheme
137 of PAMPA is shown in Fig. 2. The simplicity of the procedure and the flexibility for
138 incorporating varied lipid bilayers, including real and synthetic membranes, have made it
139 a very attractive alternative to researchers. Readers are referred to comprehensive articles
140 on the trends in PAMPA methodologies exploring distinct membranes and/or using
141 chemometrics to build suitable models [9,13,14]. In most cases, the literature studies are
142 focused on the prediction of pharmacokinetic parameters and studying the permeability
143 of varied targets through biomembrane surrogates [15–18]. Nevertheless, PAMPA-

144 related synthetic membranes have been limited so far to PVDF supports coated with
145 varied phospholipid constituents and oil membranes for gastrointestinal absorption, BBB
146 and skin [10,13]. On the other hand, other ABM methodologies have been proposed to
147 obtain more representative models of the human barrier, such as PVPA, Permeapad® and
148 AMI systems [10], as explained below.



149

150 **Fig. 2.** Scheme of the PAMPA procedure and magnification of the passive diffusion of
151 targets through lipid bilayers. Created with BioRender.com.

152 PVPA is an ABM approach that consists of incubating a liposome-laden filter support
153 that will act as a barrier mimicking the phospholipid bilayer of the intestinal cell
154 membrane [19,20]. By changing the membrane composition other specific human organs
155 could be easily simulated [21]. Recently, the incorporation of the mucus layer has been
156 introduced as an interesting alternative to standard PVPA [22–24]. This modification
157 relies on the fact that the mucus layer is the first barrier that the targets will need to cross
158 for absorption through epithelial tissues and is mimicking all mucosal surfaces in the
159 human body. For example, Calvo-Lerma *et al.* [25] combined mucus-PVPA with the *in*

160 *vitro* intestinal lipolysis model, which simulates physiological gastrointestinal conditions,
161 to study nutrient hydrolysis. In this case, the authors evaluate the permeation *in vivo*
162 (measured as the so-called area under the curve) of fenofibrate in self-nanoemulsifying
163 drug delivery systems. In this combined system, the amount of drug solubilized over time
164 during lipolysis did not correlate with the *in vivo* absorption ($R^2 < 0.4$). However, the
165 permeated amount using the mucus-PVPA methodology after lipolysis did have a strong
166 correlation with the *in vivo* data ($R^2 = 0.995$) while the mucus-PVPA permeation in the
167 absence of lipolysis was also well correlated with *in vivo* permeation ($R^2 = 0.926$). The
168 main conclusion of this work is that the use of mucus-PVPA in combination with
169 gastrointestinal fluids might offer better simulation of the human absorption conditions.

170 Permeapad® is another AMI based on the use of PC immobilized between two barriers
171 so as to avoid leaking. The PC forms lipid crystals which in the presence of water swell
172 and build a tightly packed layer of spheroids with lipid bilayers intercalated with water
173 layers as cellular membrane surrogates. Generally, Permeapad® is used in combination
174 with 96-well plate, disks for side-by-side chambers or Franz diffusion cells [10].
175 Generally, Permeapad® is aimed at evaluating drug permeability [26–28] but no
176 innovation regarding the membrane surrogate has been performed since the first
177 Permeapad® model launched in 2015 [29]. Only modifications concerning the increase
178 of the interfacial area-to-donor-volume-ratio [30] have been reported for improving the
179 correlation with rat IA against those obtained with traditional permeation systems (side-
180 by-side systems and Caco-2-cell membranes).

181 AMIs are (phospho)lipid-free permeation systems consisting of a regenerated cellulose
182 membrane barrier with a given molecular mass cut-off that is placed between two plastic
183 rings [31]. For example, a reasonable correlation was observed for poorly water-soluble
184 drugs dissolved in simulated/human intestinal fluids against the standard Caco-2

185 absorption system [32]. Also, AMI can be modified with a mucus layer for a better
186 simulation of physiological conditions [33]. In brief, AMI is a cost-effective high-
187 throughput approach to investigate passive permeation. Yet, innovations concerning AMI
188 are still to come.

189 Another *in vitro* testing assay commonly adopted in pharmacological applications for
190 mimicking passive IA is the so-called Franz-cell system [34]. Here, the donor and
191 receptor compartments are separated by the animal model membrane with the stratum
192 corneum facing the donor compartment. Franz-cell permeation systems however are
193 prone to low-reproducibility, changing of the cell model is required depending on the
194 release kinetics, and the use of the instrument is not user-friendly compared to PAMPA
195 systems. In the literature, the main applicability of Franz-cell devices relates to skin
196 permeation, rather than gastrointestinal/BBB transfer. In order to avoid ethical issues
197 associated to excised human or animal skin, replacement of those by synthetic membranes
198 such as Strat-M^{TM} have been proposed as an interesting alternative [35].

199

200 **2.2. Dynamic biomimetic systems**

201 **2.2.1. Immobilized artificial membrane (IAM) chromatography**

202 Notwithstanding the quest of biorelevant analytical methods for better *in vitro* prediction
203 of bioparameters, and the vast amount of research published in the IAM field (see Table
204 1 for an account of chromatographic systems, experimental data and *in vitro* IA
205 parameters), innovative aspects have hardly been ever considered since the launching of
206 the commercial IAM.PC.DD2 and IAM.PC.MG columns based on immobilized PC [36].

207 Usually efforts have been directed to merely apply these commercial columns with
208 standard separation methodologies to the prediction of bioparameters based on

209 chromatographic data vis-à-vis experimental values including the BBB permeability (Log
210 BB), intestine absorption values (P_{eff}), percentage of human oral absorption (%HOA),
211 and *in vitro* absorption or permeability (P_m) using the Madin-Darby kidney (MDCK) cell
212 line [37–51]. Another interesting parameter estimated with commercial IAM
213 chromatographic columns is the octanol/water partition coefficient for non-ionizable
214 compounds ($P_{o/w}$) or for ionizable compounds (D). Also, permeability data estimated
215 from PAMPA can be likewise obtained via IAM chromatographic columns [38,42,44–
216 46,48,50,52]. Although the retention factor (k) obtained from IAM columns is the most
217 common chromatographic variable for estimation of bioparameters, other experimental
218 IAM data such as CHI-IAM (chromatographic hydrophobicity index, [53]) and ΔLog k
219 (residual error of the prediction of Log k using Log P or Log D) are also used. CHI is
220 calculated from the inverse linear relationship obtained by plotting log k versus the
221 acetonitrile concentration in the mobile phase and is defined as the quotient of the
222 intercept (Log k_w, the logarithm of the retention factor with a mobile phase of 100% of
223 water or aqueous buffer) and the slope (the smaller the slope the greater is the reversed-
224 phase type interaction with the IAM sorbent). Some authors use Log k_w instead Log k
225 from IAM to predict bioparameters, although more experimental data is still necessary.
226 In addition, ΔLog k is calculated as the difference between the experimental and the
227 predicted Log k from IAM. For this purpose, the experimental Log k is plotted against
228 Log P or Log D for a set of compounds, and then the predicted log k is obtained from the
229 correlation equation. Usually, the greater the ΔLog k the lower is the log P (or D) because
230 the weaker is the interaction of the given compound with the chromatographic column
231 and thus the prediction is less accurate.

232 In IAM, a mathematical model usually based on partial least-squares is built for a set of
233 compounds utilizing a given experimental parameter from the chromatographic system,

234 i.e., log *k*, CHI or ΔLog *k* but including other molecule properties if necessary (i.e., log

235 *P*, ionization effect, number of polar groups or polar surface area, among others) against

236 *in vivo* data as illustrated in Eq. 1.

237 $$Bp = a + bP^1 + cP^2 + dP^3 \ldots \qquad \text{Eq. (1)}$$

238 **Table 1.** Review of published literature using IAM chromatography for prediction of bioparameters and study of interactions with phospholipid membrane
239 published from 2015 up to 2021.

Column[1]	Analytes	Mobile phase Technique	Comparison with	Comments	Reference
IAM PC.DD2 (c, p, pkc) Sphingo-IAM (s, p, pkc)	Drugs	MeOH/DPBS HPLC-UV	Log BB	Preparation of a sphingomyelin-based column to compare with the commercials IAM PC.DD2 and a cholesterol-based column (Cosmosil cholester). Similar predictive performance was obtained for all the columns and not improvement for the combined data.	[37]
Poly(GMA-co-EDMA)@PC (s, m, fs)	Organic acids, lidocaine, and sulfanilamide	MeOH/DPBScH capillary LC-UV	Log %AIRI	Preparation of a Soybean PC column by covalently attachment on monolithic phase thought the phosphate group for capillary LC. The results showed good relationships to predict the bioparameters selected.	[54]
Poly(MDPC-*co*-EDMA) (s, m, fs) IAM PC.DD2 (c, p, pkc)	Proteins and basic drugs	Ammonium acetate buffer/ACN nanoLC and HPLC-UV	Between them	A phosphocholine methacrylate derivative have been synthetized and copolymerized with a crosslinker to obtain a novel monolithic stationary phase. Good correlations with commercial IAM PC.DD2 column was registered.	[55]
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Acidic, basic and zwitterionic drugs	PBS HPLC-UV	Log $P_{o/w}$ Log D Log P_{eff}	The selected commercial columns were used to predict the analytes' Log P_{eff} values showing no relation using the retention factors. However, better results were obtained considering the polar and electrostatic forces.	[38]
IAM PC.DD2 (c, p, pkc)	Neutral, acidic, basic and zwitterionic drugs	PBS/ACN HPLC-UV	%HOA Log D MDK Cell	Retention indices on the commercial column were used to predict different parameters of 22 drugs, including the human oral absorption. The results showed a limited prediction ability.	[44]

			lines Log P_{eff}		
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Drugs	PBS/ACN HPLC-UV	Log BB PAMPA-BBB Log $P_{o/w}$.	Δlog k_w^{IAM} was used to predict the BBB passage and the present study demonstrates the soundness of this parameter to predict it. In addition, it showed superior capacity than PAMPA-BBB and Log $P_{o/w}$.	[45]
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Drugs	PBS HPLC-UV	Log P_{eff} Log $P_{o/w}$ Log D	Δlog k_w^{IAM} were used to predict the intestinal absorption of drugs with good results. Also, the authors interpret that polar/electrostatic forces between drugs and phospholipids play a major role in the passage through biomembranes.	[46]
IAM PC.DD2 (c, p, pkc) Poly(MDPC-*co*-EDMA) (s, m, fs) Poly(MSDPC-*co*-EDMA) (s, m, fs)	Proteins Pharmaceutical compounds	H_2O/ACN (both with 0.1% TFA) nanoLC and HPLC-UV	Between them	MCP based on phosphocholine and MDSPC based on 11-aminoundecanoic acid a phosphocholine derivative were used to act as methacrylate monomers in the preparation of monolith stationary phases. Both synthetized columns were compared with the commercial IAM column showing good correlations.	[56]
Regis IAM Fast Mini Screening (c, p, pkc)	Drugs	AAB/MeOH HPLC-UV and TOF-MS	Log BB	The commercial IAM column was used in combination with MS to predict the BBB passage obtaining solid statistics. Although, the common DPBS solvent was substituted by an AAB buffer, the predictive power was similar.	[47]

IAM PC.DD2 (c, p, pkc)	Drugs Organic compounds	AAB/ACN HPLC-UV	PAMPA MDCK cell lines	253 molecules, including few organic compounds apart from drugs were used to study the IAM commercial column to predict passive permeability obtained by PAMPA and MDCK systems. The combination of IAM data with polar surface area led to satisfactory predictions.	[48]
IAM PC.MG (c, p, pkc)	Bisphenols	PBS/CAN HPLC-UV	Log BB Skin and Corneal permeability Cell toxicity	IAM commercial column was used to stablish relationships between *in vitro* toxic activity of bisphenols and phospholipophility obtained by retention on IAM column. The results showed good correlations where more interaction with the phospholipid means more toxicity.	[49]
IAM PC.DD2 (c, p, pkc)	Penetrating and no-penetrating BB compounds	PBS/ACN HPLC-UV	Log $P_{o/w}$ P_m Plasma protein binding Log BB	The manuscript is focused on the use of IAM retention factors, PPB and permeability to predict the BBB. The results showed that more than one parameter is necessary to obtain reasonable predictions.	[50]
IAM PC.DD2 (c, p, pkc)	Perfluorinated alkylated substances	AAB/ACN UPLC-MS/MS	Cellular accumulation	Phospholipophility obtained by retention factor on the IAM commercial column was used to predict the cellular accumulation in different cell types showing high correlations.	[51]
IAM PC.DD2 (c, p, pkc)	Peptides	AAB/ACN HPLC-UV	Log BB	The chromatographic data was used to derive estimated *in vivo* distribution, drug efficiency, brain tissue binding, fraction unbound in brain and plasma, brain to plasma ratio and cell partition.	[39]
IAM PC.DD2 (c, p, pkc)	Flavonoids	H_2O/ACN HPLC-UV	Cell-based permeability		[40]

IAM PC-MG (c, p, pkc)				IAM stationary phases were used to obtain correlations between cell permeability literature data. Both stationary phases showed comparable performance towards Caco-2 cell permeability.	
IAM PC.DD2 (c, p, pkc)	Psychopharmaca	PBS/ACN HPLC-UV	Log BB	Gradient reverse elution was used to develop a linear correlation between IAM column retentions and Log BB showing extremely good results for eleven drugs.	[41]
IAM PC-MG (c, p, pkc)	Pesticides	PBS/CAN HPLC-UV	LC_{50} LD_{50}	The potential of IAM to predict ecotoxicological endpoints of 39 pesticides was evaluated. IAM retention factors showed promising predictions respect to the ecotoxicological risk	[57]
Bovine brain PS liposomes (s, fs, fs)	Drugs	40 mM HEPES CEC-UV	Log BB PAMPA-BBB	A novel *in vitro* method based on the use of liposomes in capillary electrochromatography was used to predict *in vivo* data Log BB and cell permeability.	[42]
IAM PC.DD2 (c, p, pkc)	Beyond rule of 5 molecules	AAB/ACN UV	P_m	Study of lipophilicity using an IAM column for beyond rule of 5 molecules. In addition, the obtained results were used to check the relationship with solid permeability.	[43]

240 [1] s: synthetized; c: commercial; s/c: synthetized based on a commercial column; m: monolith; p: particles; fs: fused silica; pkc: packed column

241 Abbreviations: Glycidyl methacrylate (GMA); Ethylene glycol dimethacrylate (EDMA); Phosphatidyl choline (PC); Methanol (MeOH); AAB: Ammonium acetate buffer;
242 Dulbecco's Phosphate buffer saline (DPBS); Acetonitrile (ACN); 12-methacryloyl dodecylphosphocholine (MDPC); Chromatographic hydrophobicity index (CHI); Jejenum
243 absorption values (Log P_{eff}) 1-dodecanoyl-2-(11-methacrylamidoundecanoyl)-sn-glycero-3-phosphocholine (MDSPC); Thrombin (THR); Time of flight mass spectrometry
244 (TOF-MS); Ammonium bicarbonate buffer (ABB); human oral absorption (%HOA); Madin-Darby canine kidney (MDCK); Parallel artificial membrane permeability assays
245 (PAMPA); Blood-brain-barrier (BBB); Sorption affinity into a phospholipid membrane (K_{PLIPW}); Plasma protein binding (PPB); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic
246 acid (HEPES); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); 1,2-diacyl-sn-glycero-3-phospho-l-serine (PS).

247 In this equation, the *in vivo* bioparameter (*Bp*) is explained by a constant (*a*) and a series
248 of parameters (P^n, where *n* is the number of the parameter) multiplied by its slope (*b, c,*
249 *d*, etc.). Once built, the model is applied to target analytes for estimation of the *in vitro*
250 IA parameter ($Bp_{estimated}$) of every analyte. Good correlations between Bp $_{estimated}$ and *in*
251 *vivo* BP are then sought for validation and acceptance of the model. Practically all the
252 papers in the literature focused on corroborating the utility of IAM columns for
253 pharmaceutical drugs and drug development [43], with correlations (R^2) of Log *k* from
254 IAM against *in vivo/ex vivo* parameters using real biological membranes usually ranging
255 between 0.7 and 0.8. Novel bioparameters that are proven to be appropriately estimated
256 *in vitro* by IAM include cellular accumulation (predicted by CHI-IAM) [51], cell toxicity
257 using Log *k* [49] and ecotoxicological risks using a model, which includes log *k* from
258 IAM chromatographic data and other physicochemical and molecular descriptors such as
259 hydrogen bond donor/acceptor properties, among others [57]. In the case of ΔLog *k,* an
260 inverse relationship (negative slope) against with the selected bioparameter is sought
261 because high ΔLog *k* (i.e., high residuals) usually stands for a weak interaction of the
262 compound with the biomimetic system (and expected with real membranes) while low
263 ΔLog *k* (low prediction error) is obtained for compounds with a high-sorbent interplay,
264 and thus with potential high IA.

265 It should be noted that compounds other than drugs have been scarcely studied by IAM,
266 yet some environmental pollutants such as alkylbenzenes, polycyclic aromatic
267 hydrocarbons (PAHs), bisphenols and perfluorinated alkylated substances (PFAS) have
268 been targeted to [49,51,58]. In our opinion, the use of bioinspired stationary phases to
269 estimate cellular accumulation PFAS is a promising approach in human exposomic
270 studies [51]. Literature results showed that the cellular accumulation is highly dependent
271 upon lipid binding expressed as CHI-IAM at pH 7.4 [59]. If other parameters, such as

Log *D* at pH 7.4 and Log *P* are added to CHI obtained by IAM, according to Eq. 1, the predictive results are greatly improved.

Trends in the IAM field are focused on the synthesis and testing of novel stationary phases with a variety anchored phospholipids [37,58] or the fabrication of methacrylate-based chromatographic monoliths for microscale separation by using chemically modified phospholipids with vinyl moieties to undergo UV/thermal copolymerization (Fig. 3) [54–56].

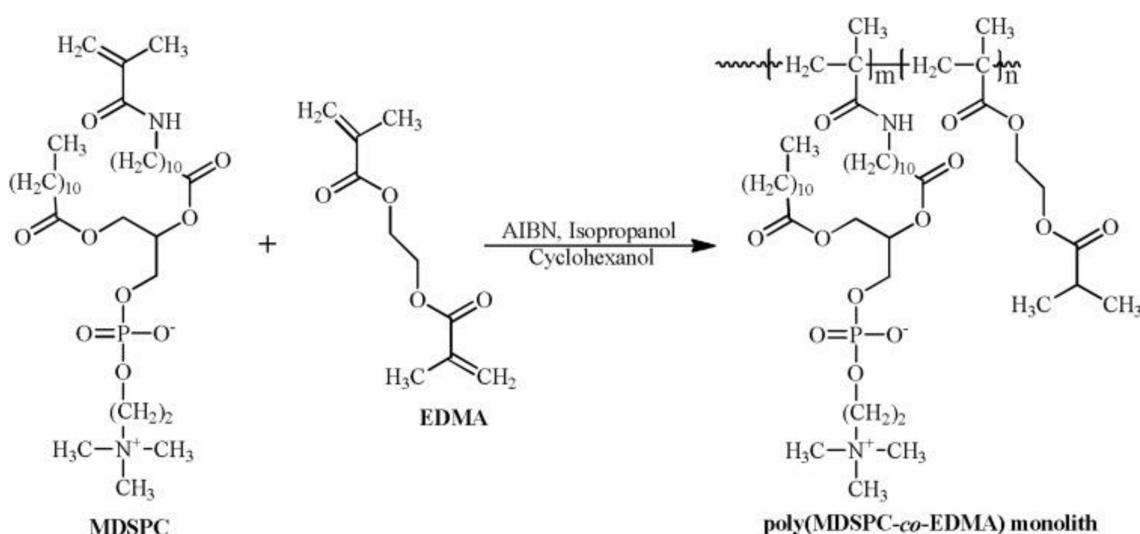


Fig. 3. Schematic diagram of the preparation of a polymer containing a modified phospholipid with vinyl groups. MDSPC: 1-dodecanoyl-2-(11-methacrylamidoundecanoyl)-sn-glycero-3-phosphocholine and EDMA: ethyleneglycoldimethacrylate. Reproduced with permission of Elsevier [56].

Special attention deserves the IAM monolith column proposed by Moravcovà *et al.* [54]. In this case, the PC was uniquely anchored through the phosphate group to the column surface, thus changing the common biomolecule orientation in IAM columns, for which the alkyl chains are usually bounded to the column surface. Similar correlations against *in vivo* parameters than those reported for with commercial columns were obtained for the studied analytes (dye, amines, anti-inflammatory, antibacterial, antifungal, analgesics,

290 and bronchodilator drugs), although no direct comparison with commercial columns was
291 performed. However, the chemical procedure for binding of PC through the polar
292 moieties seems not straightforward as compared to the facile fabrication protocols of
293 commercial IAM.PC.DD2 and IAM.PC.MG columns. Table 1 shows that phospholipid
294 monomers have been employed in all IAM dynamic approaches to generate a planar lipid
295 monolayer onto material surfaces in the attempt to simulate membrane interactions with
296 xenobiotics. Nevertheless, membranes of eukaryotic cells are constituted by phospholipid
297 bilayers in a spherical/ellipsoidal shape, which are far from being mimicked with the
298 monolayers used to date. To tackle this issue, Godyń *et al.* [42] proposed an elegant
299 solution by coating silica capillaries with 1-palmitoyl-2-oleoyl-sn-glycero-3-
300 phosphocholine (POPC) and 1,2-diacyl-sn-glycero-3-phospho-l-serine (PS)-based
301 liposomes as lipid membrane surrogates to allow for electrostatic and Van der Waals
302 interactions with the target species. However, the experimental correlations with IAM
303 data (Log k) vs Log BB and Log P_e (*in vivo*) were quite poor (R^2 = 0.426 and 0.374,
304 respectively).

305 In the design of IAM methods, researchers need to incorporate runs within a certain range
306 of pH according to the pH gradient of the gastrointestinal tract (2.0-8.0) to account for
307 potential variations across the retention factor of the target compounds [44,60]. In
308 addition, a common practice is to analyse only one analyte at a time by HPLC with
309 UV/Vis detection under isocratic conditions employing low % of organic phase. Because
310 the separation is performed under non-ideal chromatographic conditions, but appropriate
311 to trigger membrane interactions, poor peak resolution is commonly observed, and thus
312 multicomponent analysis are usually not feasible. In this sense, the use of mass
313 spectrometry detection offers the opportunity to detect several compounds
314 simultaneously. Nevertheless, attention should be paid to the HPLC conditions to avoid

315 incompatible buffers with mass spectrometry, such as PBS, and select compatible eluents
316 that could potentially mimic physiological conditions.

317

318 **2.2.2 Biopartitioning micellar chromatography**

319 Biopartitioning micellar chromatography (BMC) was proposed by Escuder-Gilabert *et*
320 *al.* in 2004 [61]. BMC can be described as a chromatographic method in which the mobile
321 phase is composed by a surfactant system (commonly Brij35, non-ionic surfactant with
322 hydroxyl moieties) over its critical micellar concentration, with the surplus of monomers
323 being adsorbed onto the C18/C8 bed of a reversed-phase column to create a C18/C8-
324 surfactant bilayer. The double equilibria generated between the compound(s) and the
325 micelles (acting as pseudostationary phases) and the stationary phase surface bilayer
326 (surfactant +C18/C8 chains) that mimics both the polar and hydrophobic regions of the
327 lipid membranes are expected to simulate closely those interactions occurring in *in vivo*
328 oral absorption of drugs, BBB penetration or intestinal absorption permeability, among
329 other processes [62]. However, BMC, apart from poor column efficiency and the weak
330 solvent strength of micellar eluents, only capitalizes upon passive diffusion and therefore
331 if other underlying mechanisms are involved (*e.g.* via paracellular route or active
332 transport), large deviations can be observed. Therefore, BMC, in some instances, only
333 can provide a limited insight into the actual drug absorption in humans and biota. A
334 selection of the most interesting publications and estimated bioparameters using BMC
335 within the time span of 2015-mid 2021 are summarized in Table 2.

336 C18 reversed-phase columns have been the common choice of stationary phases [62–64]
337 because the C18 chains foster interactions by Van der Waals forces with the alkyl chains
338 of the surfactant monomers generating the bespoke surface bilayer. However, some

339 authors recommended alternative stationary phases, such as cyanopropyl [65] or
340 aminopropyl [66] attempting to obtain more polar surfaces for low-retained analytes in
341 C18 columns. For example, De Vrieze *et al.* [67] combined a classical C18 column with
342 synthetized miltefosine which was used as a surfactant in BMC for a better mimicry of
343 biological membranes than those obtained by other surfactant counterparts to predict HIA
344 and log BB values. In that work, for example, PLS was used to predict Log BB using the
345 Log *k* obtained from BMC and other molecular descriptors as shown in Eq. (2)

346 $$\text{Log BB} = -2.669 + 0.234 \times \text{Log } k + 0.699 \times \alpha - 0.048 \times \text{P} - 0.002 \times \text{WS}_{7.4} + 0.009 \times \text{PB}$$
347 $$+ 0.034 \times \text{HIA} - 0.017 \times \text{PSA} + 0.167 \times \text{HBA} \quad \text{Eq. (2)}$$

348

349 **Table 2.** Review of published literature using BMC technique for prediction of bioparameters published from 2015 up to 2021.

Column[1]	Analytes	Mobile phase Technique	Comparison with	Comments	Reference
C18 (s/c, p, pkc)	Drugs	Brij-35 in PBS HPLC-UV	LC50	A two-dimensional liquid chromatography method was developed using a BMC separation in first dimension and C18 reversed phase in the second dimension to study the identification, bioactivity and toxicity of drugs with a time-saving and low-cost system. The second dimension improve the weak separation ability of BMC.	[63]
C18 (c, p, pkc)	Drugs	Miltefosine aqueous solution TOF-MS	Log BB HIA	A synthesized surfactant (miltefosine) that mimics better the biological layers has been used for BMC. The retention factors in combination with other descriptors were used to develop models to predict Log BB and HIA and the correlation coefficients were between 0.37 and 0.88.	[67]
C8 (c, p, pkc)	Drugs	PC and SDS HPLC-UV	Log D	The use of microemulsions in presence of mixed micelles and oil has been used to mimic the biomembrane. The system showed better correlations for log *D* than other IAM chromatograpy. However, the authors did not use the system to predict other bioparameters.	[68]
Cyanopropyl column (c, p, pkc)	Drugs	NaDC aqueous solution HPLC-UV	HIA	Alternative BMC system using bile salts have been used to predict intestine permeability expressed as HIA for pharmaceutical compounds obtaining r^2 between 0.75 and 0.86.	[65]
C18 (s/c, p, pkc)	Structurally unrelated analytes	SDS aqueous solution HPLC-UV	Log BB	Partial least square method was used to predict BBB using retention factors of BMC and other topological and physicochemical parameters. The results showed high correlations (r^2 = 0.83). Also, when IAM columns were used (r^2 = 0.78).	[64]

Zorbax Extend-C18 (c, p, pkc)	IRs/α-Ars, drugs	Brij-35 in PBS HPLC-UV	Log BB	BMC retention factors were used to estimate the BBB permeability of different drugs. The correlations of BMC showed higher correlation factors ($r^2 = 0.77$) than common reversed-phase ($r^2 = 0.58$).	[62]
APS (c, p, pkc)	Drugs	NaDC aqueous solution HPLC-UV	HIA	In this study the prediction of HIA was extended to more compounds thanks to the incorporation of an aminopropyl column. The micelle-water partition coefficients were calculated and combined with other descriptors and used to predict HIA showing correlations (r^2) in the range 0.72-0.85.	[66]
- (c/s, -, fs)	Drugs	Brij35, Tris and HEPES HPLC-UV	Log BB	Biopartitioning micellar electrokinetic chromatography (BMEKC) as alternative to common BMC was used to estimate the BBB of drug candidates. The proposed methodology showed similar correlation coefficients ($r^2 = 0.73$) compared to that found on conventional BMC ($r^2 = 0.75$)	[69]

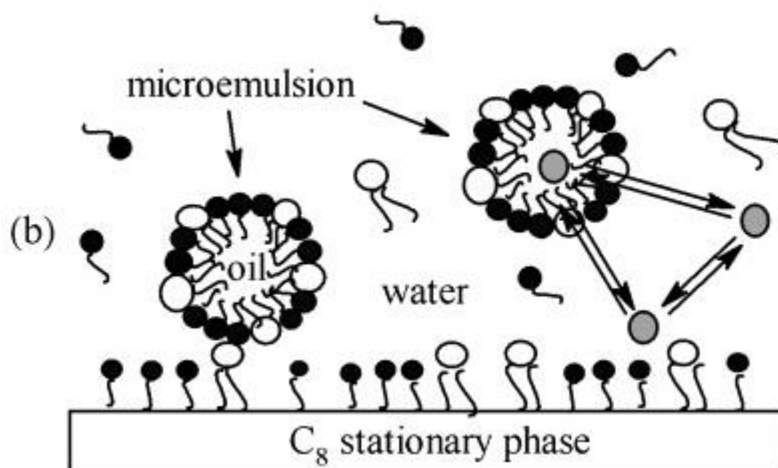
350 [1] s: synthetized; c: commercial; s/c: synthetized based on a commercial column; m: monolith; p: particles; fs: fused silica; pkc: packed column

351 Abbreviations: Human intestinal absorption (HIA), sodium deoxycholate (NaDC), β-hydroxy-β-arylalkanoic acids (HAA), Quantitative Structure-Retention Relationship
352 (QSRR), imidazoline/α-adrenergic receptor (IRs/α-ARs), anionic sodium dodecyl sulfate (SDS), reversed- phase aminopropyl column (APS)

353

354 in which α is the total molar charge at pH 7.4, P is the polarizability, WS7.4 is the aqueous
355 solubility at pH 7.4, PB is the plasma protein binding, HIA is the human intestinal
356 absorption, PSA is the polar surface area and HBA is the hydrogen bond acceptor capacity
357 for every target compound. The authors investigated 36 drugs, and good correlation
358 coefficients for predicted log BB against *in vivo* log BB were obtained ($R^2 > 0.72$), yet
359 not only chromatographic and physicochemical parameters from the literature but *in vivo*
360 data such as HIA were needed to build a reliable model. The relevance of this work is the
361 usage of a phosphocholine-based surfactant as a model of cell membrane because
362 phospholipids cannot be used as reliable surfactants because of solubility issues.
363 However, as can be seen by the reported results, the intricate interactions in live
364 organisms cannot be explained merely by the underlying passive diffusion in BMC, even
365 with phosphocholine-based surfactants. Other authors proposed hybrid micelles of PC
366 and sodium dodecyl sulfate (SDS) in the presence of an organic phase (mixture of *n*-
367 butanol and ethyl acetate) that are aimed at predicting biomimetic parameters by
368 microemulsion liquid chromatography (MELC) [68]. The three-phase
369 (microemulsion/water/column) model (Fig. 4) features better prediction of Log *D* than
370 that obtained by IAM chromatography. Notwithstanding the fact that the authors
371 suggested that permeability descriptors can be appropriately described with MELC
372 (determined by principal components analysis), potential correlations between *in vivo* and
373 MELC data were regrettably not investigated.

374



375 **Fig. 4.** Schematic representation of proposed MELC interphase using C8 stationary
376 phase and a microemulsion constituted by PC (white circles), SDS (black circles) and
377 an oil. The target compound is represented in grey color. Reproduced with permission
378 of Elsevier [68].

379 In another work, Waters *et al.* [65] exploited sodium deoxycholate (bile salt) and the
380 reversed phase cyanopropyl column to predict HIA for various drugs (R^2 = 0.75-0.86). In
381 the same way, Shokry *et al.* [66] selected the same bile salt in combination with an
382 aminopropyl column to study a larger number of compounds with contrasting behaviors
383 related to the affinity to the micelle based on hydrophilic/lipophilic interactions. The
384 micelle-water partition coefficients were calculated and showed that antibinding
385 compounds (to the micelle) have better retention onto the stationary phase with the
386 increase of surfactant concentration while non-binding compounds do not show alteration
387 of their retention times with changes in micelle concentration. The partition coefficients,
388 in combination with other descriptors such as molar volume and aqueous solubility, were
389 used to predict HIA with relatively good results against *in vivo* HIA (R^2 = 0.72-0.85).
390 However, the two columns described in this paragraph showed similar correlations
391 against *in vivo* HIA for an alike pool of drugs thus demonstrating that both micelle

392 systems in combination with reversed-phase stationary phases bearing polar moieties are
393 biorelevant.

394 In our opinion, BMC has made tremendous strides recently to leverage its main
395 advantages: (i) simultaneous simulation of a number of pharmacokinetic parameters with
396 a single measurement, (ii) data robustness, (iii) low cost, (iv) green credentials, and (v)
397 flexibility in the selection of the stationary phase and surfactants. However, gradient
398 conditions are herein excluded, with the consequent increase of the analysis time under
399 isocratic conditions [70]. BMC fundamentals have been also incorporated in other
400 separation techniques, such as micellar electrokinetic chromatography (MEKC). In this
401 electroseparation technique, the pseudo-stationary phase added to the background
402 electrolyte consists of an aqueous surfactant solution. In fact, MEKC and BMC can be
403 synergistically combined in the so-called biomimetic MEKC (BMEKC) in which the
404 electrophoretic conditions simulate the interaction between analytes and biological
405 membranes [69]. The BMEKC system proposed by Ciura *et al.* [69] provides a similar
406 correlation coefficient than that reported by BMC methods for *in vitro* BBB evaluation
407 (R^2 = 0.73 against *in vivo* BBB) but with significantly lower consumption of reagents
408 thus coping with green analytical chemistry principles.

409

410 **2.2.3 Immobilized plasma protein chromatography**

411 IPPC is a chromatographic technique capitalizing upon the measurement of protein
412 binding throughout the cell membrane or the bloodstream using stationary phases
413 modified with proteins such as human serum albumin (HSA) or alpha-1-acid glycoprotein
414 (AGP) [5] among others. The interaction of the target compounds with proteins is an
415 important bioparameter inasmuch as their pharmacodynamic behaviors, cell permeation

416 and drug-drug interactions might be significantly altered. Up to the date, CHIRALPACK
417 HSA and its counterpart of AGP have been the common stationary phases to measure the
418 protein binding for different analytes such as drugs [39,71], BBB and non-BBB
419 penetrating compounds [50], phytoestrogens [72] and bisphenol analogues [73]. In these
420 cases, the majority of the works reported binding values or their comparison with Log *P*.
421 However, Valko *et al.* [71] elegantly attempted to estimate the steady state volume of
422 distribution (V_{dss}) of target drugs, which is a key parameter for setting drug doses, by
423 using log *k* from HSA column (related to the binding protein value) and also from IAM
424 column (membrane permeation) to predict the *in vivo* V_{dss}, although low correlations were
425 observed between *in vivo* and chromatographic data (R^2 = 0.56-0.66). Therefore, some
426 authors proposed new column materials to measure protein binding values *in vitro*. As an
427 example, Ma *et al.* [74] fabricated a frontal silica-based affinity chromatographic column
428 with hierarchical mesopores and penetrable macropores containing covalently attached
429 BSA by Schiff base. The columns enabled the enantioseparation of D/L tryptophan and
430 the frontal affinity chromatographic analysis of imatinib mesylate and demonstrated that
431 there is a single type of binding between the analyte(s) and BSA. The authors signaled
432 that these columns will open new avenues for the measurement of the protein-drug
433 interaction of low-to-moderate retained analytes in other chromatographic materials. In
434 another interesting example, Liang *et. al.* [75] immobilized angiotensin II type I receptor
435 (AT1R) to probe antihypertensive compounds. For this purpose, the AT1R was expressed
436 using *E. coli* and after cell lysate, the protein was immobilized onto 6-chlorocaproic acid-
437 activated amino polystyrene microspheres. The as-prepared columns were used for
438 elucidating drug-receptor binding kinetics and thermodynamic parameters. The authors
439 expanded the use of the AT1R columns for the identification of puerarin and rosmarinic
440 acid as antihypertensive compounds in natural products although both species are far

441 away from the requirements of a drug candidate. In short, IPCC has been mostly linked
442 to the use of the common HSA and AGP columns, which are readily available. However,
443 it is necessary to prepare novel phases that offer better interaction profiles to obtain a
444 deeper understanding of the involved processes in biological systems. In fact, there is a
445 quest of novel IPCC columns containing other proteins, such as globulins,
446 phosphoproteins, lipoproteins and/or C-reactive protein to account for a broad range of
447 analyte-protein interactions. In addition, the synergetic combination of various proteins
448 in a single material may contribute to unveil the underlying mechanisms in IA processes.

449

450 **2.2.4 Miscellaneous biomimetic systems**

451 Apart from standard biomimetic structures encompassing PC, proteins, and surfactant
452 micelles as artificial membranes, other biorelevant systems have been used to elucidate
453 the complex interactions occurring between the target compounds and the lipid
454 membrane. For example, Stephen *et al.* [76] harnessed cellular membrane affinity
455 chromatography (CMAC) which consists of the immobilization of cell membrane
456 fragments with fully functional transmembrane proteins onto IAM stationary phases. The
457 early usage of these stationary phases was limited to the binding characteristics of
458 immobilized transmembrane proteins. However, as demonstrated in that work, CMAC
459 can be expanded to the identification of pharmacologically active metabolites from
460 natural products. In any case, CMAC columns do not serve as universal tool in drug
461 discovery but do speed up the process of target identification. The bioanalytical and
462 pharmacological potential of these columns is still in its infancy, but we do expect the
463 upward trend of CMAC in IPPC and IAM to continue in the foreseeable future.

464 Notwithstanding the fact that liquid chromatography has been by far the separation
465 technique of choice for resembling biological processes *in vitro*, other dynamic
466 techniques such as supercritical fluid chromatography (SFC) [77] and thin-layer
467 chromatography (TLC) [78] have been also adopted to predict bioparameters, namely,
468 permeation through BBB. For example, Russo *et al.* [77] used SFC to estimate the PSA
469 of target compounds, and in combination with other parameters (viz, IAM retention
470 factor, water accessible surface and number of aliphatic carboxylic acids and
471 phenol/enol/carboxyl/hydroxy groups, see eq. 1) succeeded in predicting Log BB of
472 sixty-nine acid, base, neutral and amphoteric substances with good correlations with *in*
473 *vivo* Log BB data (R^2 = 0.81). Log BB can be also predicted using the chromatographic
474 data (Rf) obtained from a reversed-phase C18 TLC separation [78]. In addition, the
475 combination of Rf with PSA was suggested as a universal predictor of brain absorption
476 on the basis of excellent correlations with *in vivo* BB data (R^2 = 0.9). In summary,
477 different separation systems, including SFC, TLC and the aforementioned BMECK [69]
478 can be harnessed to the prediction of Log BB, which thus is not exclusively dependent on
479 *in vitro* data by high performance liquid chromatography.

480

481 **3. Conclusion and outlook**

482 In this review, the state-of-the-art of artificial membranes in (bio)analytical applications
483 has been critically dissected. The research developments since 2015 up to mid-2021 in
484 terms of material science have been rather limited and the majority of the publications
485 continue employing standard/customary methodologies (*e.g.* PVDF coated supports) or
486 commercial systems (e.g. IAM.PC.DD2 column). In the case of static artificial
487 membranes, trends are focused on the combination of mucus layers with PVPA systems

[22] and gastrointestinal fluids that led to high correlations with *in vivo* data. On the other hand, some innovative methods have been reported in dynamic modes (IAM chromatography, BMC and IPPC) aimed at ameliorating IA results. For example, novel materials for IAM chromatography have been prepared by surface attached phospholipids [54]. The idea behind is to improve the *in vivo/in vitro* correlations of bioavailability values obtained with commercial columns The incorporation of novel choline-based surfactants [67] and dedicated surfactants such as bile salts [65] have been the most interesting trends in BMC to improve IA predictions. Nevertheless, the passive diffusion through the lipid membrane mimicked by AIM and BMC is not sufficient to simulate the intricate interactions occurring in cell membranes during the absorption of compounds. For this reason, IPPC can be used to simulate other membrane-target interactions such as protein binding using columns with immobilized AGP. However, other proteins such as AT1PR has been attached to the stationary phase [75] which demonstrates the fact that IPPC is not only limited to standard membrane/serum proteins but other specific interactions with other proteins and biomolecules can be explored.

To shed light into the complex phenomena of IA, interest has grown on alternative techniques such as CMAC which uses cell membrane fragments [76], BMECK [69], MELC [68], SFC [77] and TLC [78] to leverage the possibilities offered by liquid chromatographic methods. To the best of our knowledge, most of the studies dealing with artificial membranes focused on the absorption of pharmaceutical compounds, yet the IA of legacy and emerging contaminants has been scarcely studied.

Our vision is that the development of hybrid/smart materials involving monoliths, nanomaterials, metal organic frameworks and/or 3D printed templates in combination with biomolecules or membrane surrogates is expected to open new avenues for

512 mimicking the human absorption/IA of targets on account of the plethora of interaction
513 mechanisms available that resemble those of the eukaryote cell membranes.

514

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525

526

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840

841 **Figure Captions**

842 **Fig. 1.** Scheme of the different pathways for endogenous and xenobiotic compounds to
843 pass through the lipid membrane. Passive diffusion (A); facilitated diffusion (B); active

844 transport (C); endocytosis-facilitated process (D). Created with BioRender.com.

845 **Fig. 2.** Scheme of the PAMPA procedure and magnification of the passive diffusion of
846 targets through lipid bilayers. Created with BioRender.com.

847 **Fig. 3.** Schematic diagram of the preparation of a polymer containing a modified
848 phospholipid with vinyl groups. MDSPC: 1-dodecanoyl-2-(11-
849 methacrylamidoundecanoyl)-sn-glycero-3-phosphocholine and EDMA:
850 ethyleneglycoldimethacrylate. Reproduced with permission of Elsevier [56].

851 **Fig. 4.** Schematic representation of proposed MELC interphase using C8 stationary phase
852 and a microemulsion constituted by PC (white circles), SDS (black circles) and an oil.
853 The target compound is represented in grey color. Reproduced with permission of
854 Elsevier [68].

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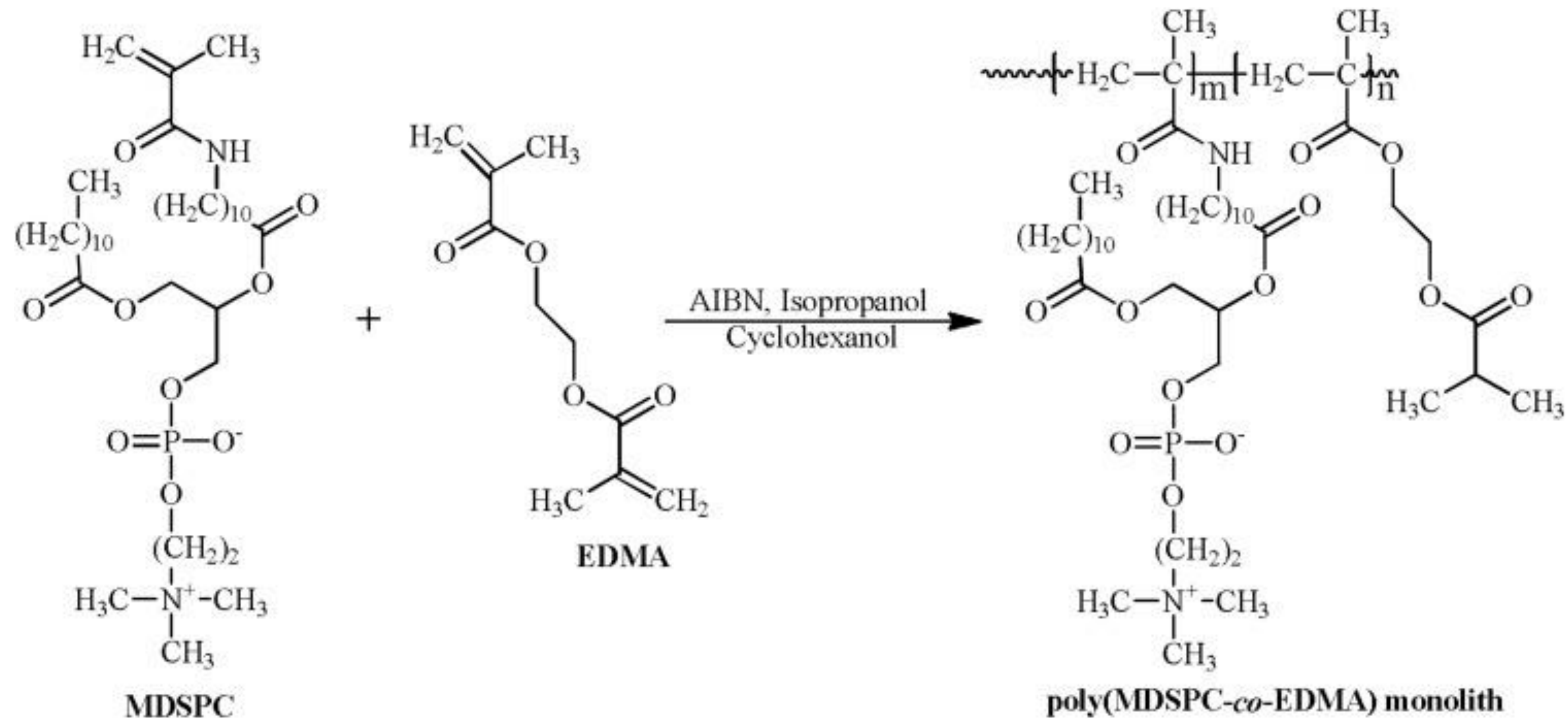

Fig. 3– Carrasco-Correa *et al.*

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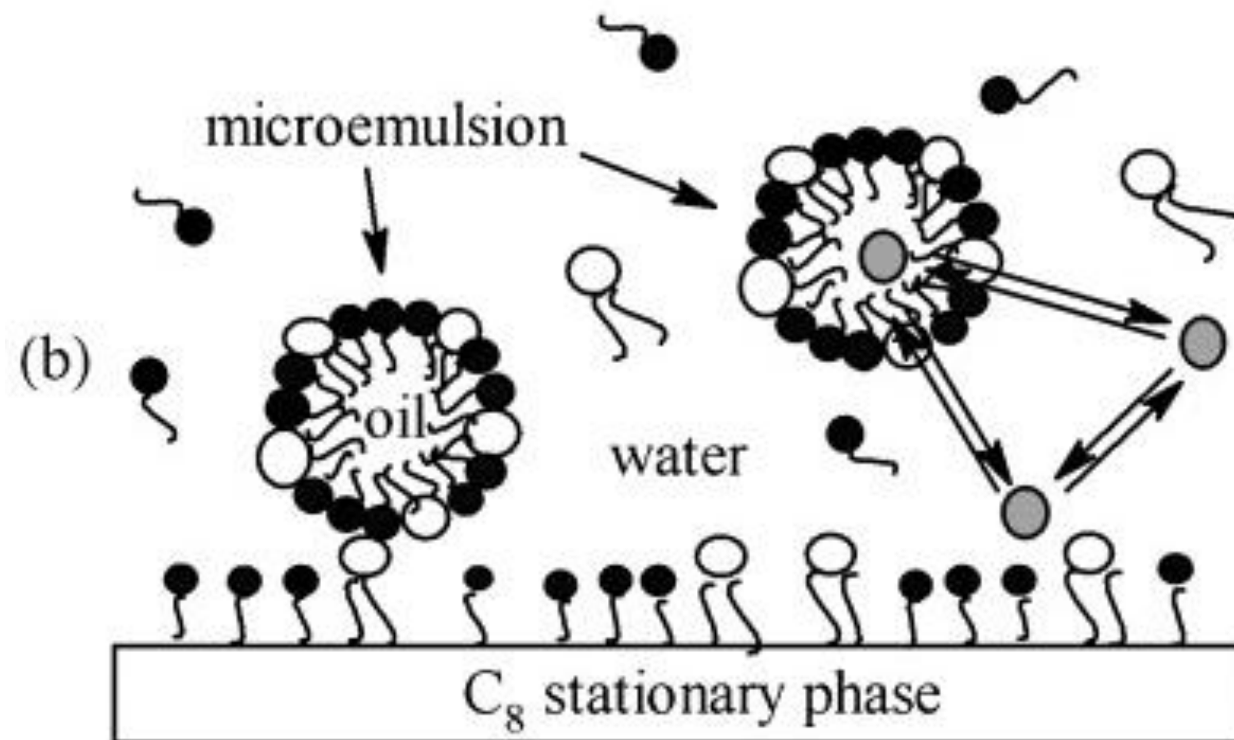

Fig. 4 – Carrasco-Correa *et al.*

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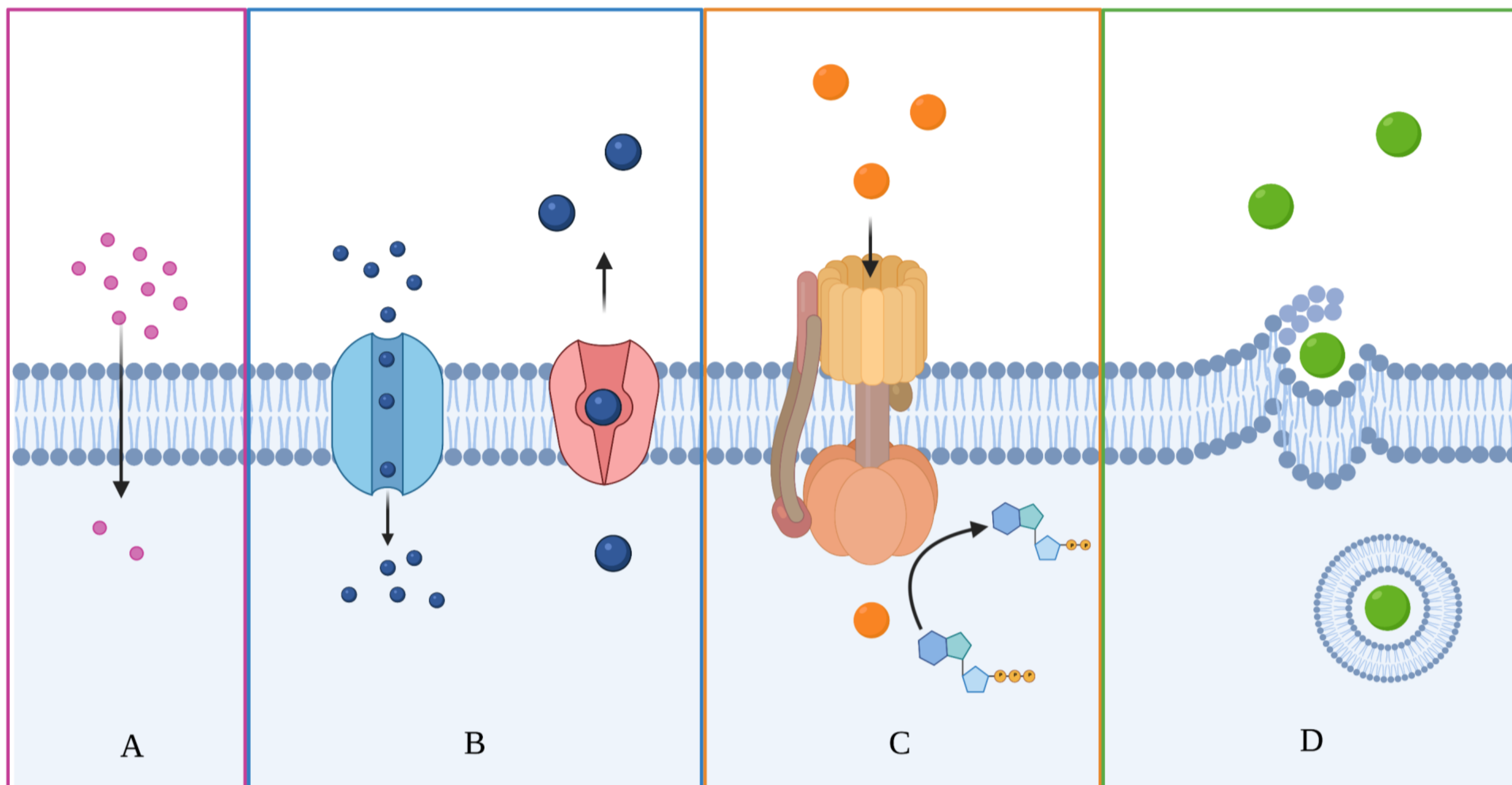

Fig. 1– Carrasco-Correa *et al.*

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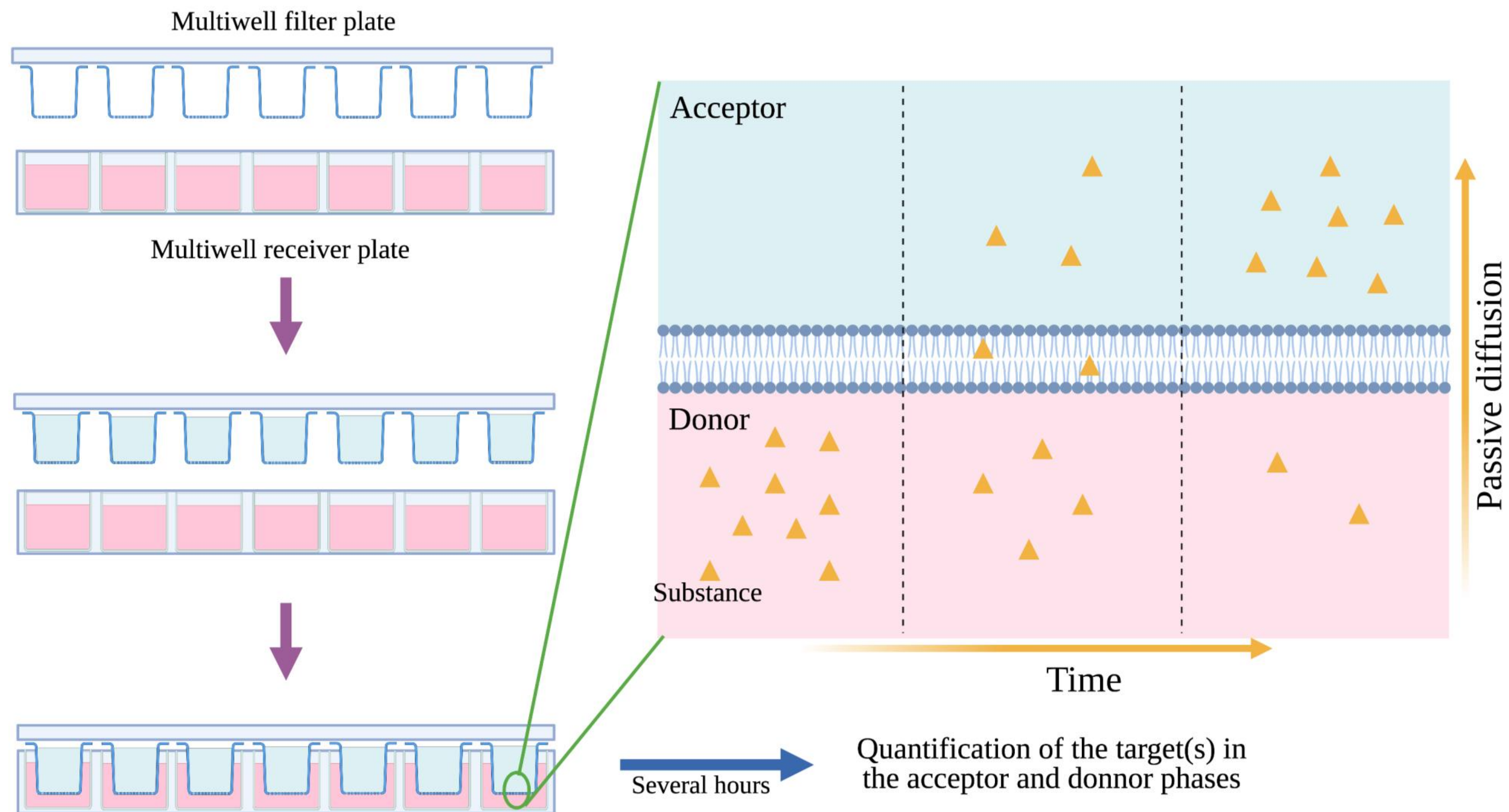

Fig. 2– Carrasco-Correa *et al.*

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Table 1. Overview of representative literature (since 2015 up to mid-2021) using IAM chromatography for prediction of bioparameters and study of interactions of targets with an immobilized phospholipid membrane.

Column[1]	Analytes	Mobile phase Technique	Bioparameters/ Comparison with	Comments	Reference
IAM PC.DD2 (c, p, pkc) Sphingo-IAM (s, p, pkc)	Drugs	MeOH/DPBS HPLC-UV	Log BB	Preparation of a sphingomyelin-based column and comparison with the commercially available IAM PC.DD2 and a cholesterol-based column (Cosmosil cholester). Similar predictive performance was obtained for all the columns but without improvement for the combined data.	[37]
Poly(GMA-co-EDMA)@PC (s, m, fs)	Organic acids, lidocaine, and sulfanilamide	MeOH/DPBS capillary LC-UV	Log %AIRI	Preparation of a soybean PC column by covalent attachment on a monolithic phase through the phosphate group for capillary LC. Good correlations were found for the prediction of the bioparameters selected.	[54]
Poly(MDPC-*co*-EDMA) (s, m, fs) IAM PC.DD2 (c, p, pkc)	Proteins and basic drugs	Ammonium acetate buffer/ACN nanoLC and HPLC-UV	Between columns	A phosphocholine methacrylate derivative was synthetized and copolymerized with a crosslinker to obtain a novel monolithic stationary phase. Good correlations with commercial IAM PC.DD2 column were reported.	[55]
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Acidic, basic and zwitterionic drugs	PBS HPLC-UV	Log $P_{o/w}$ Log D Log P_{eff}	The selected commercial columns were used to predict the Log P_{eff} values of the analytes showing no relationship with the retention factors. However, better results were obtained when incorporating polar and electrostatic forces in the model.	[38]
IAM PC.DD2 (c, p, pkc)	Neutral, acidic, basic and zwitterionic drugs	PBS/ACN HPLC-UV	%HOA Log D MDK Cell lines Log P_{eff}	Retention indices on the commercial column were used to predict different parameters of 22 drugs, including the human oral absorption. The results showed a limited prediction ability.	[44]

IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	drugs	PBS/ACN HPLC-UV	Log BB PAMPA-BBB Log $P_{o/w}$.	Δlog k_w^{IAM} was used to predict the BBB passage and this study demonstrates the soundness of this equation for reliable prediction. It showed superior prediction capacity than PAMPA-BBB and Log $P_{o/w}$.	[45]
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Drugs	PBS HPLC-UV	Log P_{eff} Log $P_{o/w}$ Log D	Δlog k_w^{IAM} was used to predict the intestinal absorption of drugs with good results. The authors suggested that polar/electrostatic forces between drugs and phospholipids play a major role in the passage through biomembranes.	[46]
IAM PC.DD2 (c, p, pkc) Poly(MDPC-*co*-EDMA) (s, m, fs) Poly(MSDPC-*co*-EDMA) (s, m, fs)	Proteins Pharmaceutical compounds	H_2O/ACN (both with 0.1% TFA) nanoLC and HPLC-UV	Between columns	MDPC based on phosphocholine and MDSPC based on 11-aminoundecanoic acid a phosphocholine derivative were used as methacrylate monomers for the preparation of monolithic stationary phases. Both synthetized columns were compared with the commercial IAM column showing good correlations.	[56]
Regis IAM Fast Mini Screening (c, p, pkc)	Drugs	AAB/MeOH HPLC-UV and TOF-MS	Log BB	The commercial IAM column was used in combination with MS to predict the BBB passage and solid statistics were obtained. Although the common DPBS solvent was substituted by an AAB buffer, the predictive power was similar.	[47]
IAM PC.DD2 (c, p, pkc)	Drugs Organic compounds	AAB/ACN HPLC-UV	PAMPA MDCK cell lines	253 molecules, including few organic compounds apart from drugs were used to study the feasibility of the IAM commercial column to predict passive permeability obtained in-vitro by PAMPA and MDCK systems. The combination of IAM data with polar surface area led to satisfactory predictions.	[48]

IAM PC.MG (c, p, pkc)	Bisphenols	PBS/ACN HPLC-UV	Log BB Skin and Corneal permeability Cell toxicity	IAM commercial column was used to set relationships between *in vitro* toxic activity of bisphenols and phospholipophility obtained by retention on IAM column. The results showed good correlations for which stronger interaction with the phospholipid indicates more toxicity.	[49]
IAM PC.DD2 (c, p, pkc)	Penetrating and no-penetrating BB compounds	PBS/ACN HPLC-UV	Log $P_{o/w}$ P_m Plasma protein binding Log BB	The manuscript is focused on the use of IAM retention factors, PPB and permeability to predict the BBB. The results showed that more than one parameter is necessary to obtain reasonable predictions.	[50]
IAM PC.DD2 (c, p, pkc)	Perfluorinated alkylated substances	AAB/ACN UPLC-MS/MS	Cellular accumulation	Phospholipophility obtained by retention factor on the IAM commercial column was used to predict the cellular accumulation in different cell types. High correlations are shown.	[51]
IAM PC.DD2 (c, p, pkc)	Peptides	AAB/ACN HPLC-UV	Log BB	The chromatographic data were used to estimate *in vivo* drug distribution, drug efficiency, brain tissue binding, fraction unbound in brain and plasma, brain to plasma ratio and cell partition.	[39]
IAM PC.DD2 (c, p, pkc) IAM PC-MG (c, p, pkc)	Flavonoids	H_2O/ACN HPLC-UV	Cell-based permeability	IAM stationary phases were used to obtain correlations with cell permeability literature data. Both stationary phases showed comparable performance towards Caco-2 cell permeability.	[40]
IAM PC.DD2 (c, p, pkc)	Psychopharmacs	PBS/ACN HPLC-UV	Log BB	Gradient elution was used to develop a linear correlation between IAM column retention factors and Log BB showing extremely good results for eleven drugs.	[41]
IAM PC-MG (c, p, pkc)	Pesticides	PBS/ACN HPLC-UV	LC_{50} LD_{50}	The potential of IAM to predict ecotoxicological endpoints of 39 pesticides was evaluated. IAM retention factors showed promising predictions towards ecotoxicological risk	[57]

Bovine brain PS liposomes (s, fs, fs)	Drugs	40 mM HEPES CEC-UV	Log BB PAMPA-BBB	A novel *in vitro* method based on the use of liposomes in capillary electrochromatography was used to predict *in vivo* Log BB and cell permeability data.	[42]
IAM PC.DD2 (c, p, pkc)	Drugs	AAB/ACN UV	P_m	Study of lipophilicity using an IAM column for drugs. The obtained results were used to check the relationship with permeability.	[43]

[1] s: synthetized; c: commercial; s/c: synthetized based on a commercial column; m: monolith; p: particles; fs: fused silica; pkc: packed column

Abbreviations: Glycidyl methacrylate (GMA); Ethylene glycol dimethacrylate (EDMA); Phosphatidyl choline (PC); Methanol (MeOH); AAB: Ammonium acetate buffer; Absorption of inverted rat intestine (AIRI); Dulbecco's Phosphate buffer saline (DPBS); Acetonitrile (ACN); 12-methacryloyl dodecylphosphocholine (MDPC); Jejenum absorption values (Log P_{eff}); Permeability (P_m); 1-dodecanoyl-2-(11-methacrylamidoundecanoyl)-sn-glycero-3-phosphocholine (MDSPC); Thrombin (THR); Time of flight mass spectrometry (TOF-MS); Ammonium bicarbonate buffer (ABB); human oral absorption (%HOA); Madin-Darby canine kidney (MDCK); Parallel artificial membrane permeability assays (PAMPA); Blood-brain-barrier (BBB); Plasma protein binding (PPB); Lethal dose 50% (LD_{50}); Lethal concentration 50% (LC_{50}); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); 1,2-diacyl-sn-glycero-3-phospho-l-serine (PS).

Table 2 Click here to access/download;Table;Table 2_Carrasco-Correa et al.docx

Table 2. Overview of representative literature using BMC for prediction of bioparameters since 2015 until mid- 2021.

Column[1]	Analytes	Mobile phase Technique	Bioparameter	Comments	Reference
C18 (s/c, p, pkc)	Drugs	Brij-35 in PBS HPLC-UV	LC_{50}	A two-dimensional liquid chromatography method was developed using a BMC separation in the first dimension and C18 reversed phase in the second dimension to study the identification, bioactivity and toxicity of drugs using a time-saving and low-cost system. The second dimension improves the weak separation ability of BMC.	[63]
C18 (c, p, pkc)	Drugs	Miltefosine aqueous solution TOF-MS	Log BB HIA	A synthesized surfactant (miltefosine) that mimics better the composition of biological layers has been used for BMC. The retention factors in combination with other descriptors were used to build models to predict Log BB and HIA and the correlation coefficients were between 0.37 and 0.88.	[67]
C8 (c, p, pkc)	Drugs	PC and SDS HPLC-UV	Log D	The use of microemulsions in the presence of mixed micelles and oil has been used to mimic the biomembrane. The system showed better correlations for log *D* than IAM chromatographic counterparts. However, the authors did not harness the system to predict other bioparameters.	[68]
Cyanopropyl column (c, p, pkc)	Drugs	NaDC aqueous solution HPLC-UV	HIA	Alternative BMC system using bile salts has been used to predict intestine permeability expressed as HIA for pharmaceutical compounds obtaining R^2 between 0.75 and 0.86.	[65]
C18 (s/c, p, pkc)	Structurally unrelated analytes	SDS aqueous solution HPLC-UV	Log BB	Partial least square method was used to predict BBB using retention factors of BMC and other topological and physicochemical parameters. The results showed high correlations ($R^2 = 0.83$), also for IAM columns ($R^2 = 0.78$).	[64]
Zorbax Extend-C18	IRs/α-Ars, drugs	Brij-35 in PBS	Log BB	BMC retention factors were used to estimate the BBB permeability of different drugs. BMC features higher	[62]

(c, p, pkc)		HPLC-UV		correlation factors (R^2 = 0.77) than those obtained with reversed-phase without micellar medium (R^2 = 0.58).	
APS (c, p, pkc)	Drugs	NaDC aqueous solution HPLC-UV	HIA	The prediction of HIA was extended to a large number of compounds thanks to the incorporation of an aminopropyl column. The micelle-water partition coefficients were calculated and combined with other descriptors for predicting HIA. Correlations (R^2) in the range 0.72-0.85 were obtained.	[66]
- (c/s, -, fs)	Drugs	Brij35, Tris and HEPES HPLC-UV	Log BB	Biopartitioning micellar electrokinetic chromatography (BMEKC) as alternative to BMC was used to estimate the BBB of drug candidates. The proposed methodology however showed similar correlation coefficients (R^2 = 0.73) than those of conventional BMC (R^2 = 0.75)	[69]

[1] s: synthetized; c: commercial; s/c: synthetized based on a commercial column; p: particles; fs: fused silica; pkc: packed column

Abbreviations: LC_{50}: Lethal concentration 50; BBB: blood brain barrier; Human intestinal absorption (HIA), sodium deoxycholate (NaDC), Quantitative Structure-Retention Relationship (QSRR), imidazoline/α-adrenergic receptor (IRs/α-ARs), anionic sodium dodecyl sulfate (SDS), reversed- phase aminopropyl column (APS)

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30th July 2021

Dear Prof. Stig Pedersen-Bjergaard,

The authors have not conflict of interest in the preparation of this review regarding the use of artificial membranes in (bio)analytical field and its potential for bioavailability studies.

Enrique Javier Carrasco-Correa