WP2.1. Targeting and crossing complex cellular membranes (Jungwirth, Jahn, Lazar, and Bouř)

Research aims

- To develop a faithful computational model of the endothelial glycocalyx verified by experiments.
- To design novel strategies for therapeutical targeting the cell glycocalyx to control the progression of metastasis without compromising cardiovascular functions.
- To improve the targeting itself by elucidating new ways enabling drugs to reach selectively their action sites via efficiently crossing the cellular membrane with its extracellular matrix.

Research plan and methodology

It is known that cells adapt to new environment by changing their metabolism. If we can change the way that the cell feels the environment, then we can either enhance or shut down various metabolic pathways. The cell feels the environment through the plasma membrane and its cover, i.e., the glycocalyx. These two elements which we aim to target by modifying their structure are at the core of this proposal.

In this project we will employ computer simulations, i.e., molecular dynamics, to obtain insight into the molecular structure of the glycocalyx where experimental studies are greatly restricted due to its dynamic and fragile nature. In concert, experimental approaches, ranging from spectroscopic techniques (such as fluorescence microscopy or vibrational circular dichroism and Raman optical activity measurements) to synthetic organic chemistry and cell biology, will also be employed primarily on smaller structural units forming the glycocalyx and crucial species interacting with it.

This project is based on the highly plausible scenario that the endothelial glycocalyx structure can play a key role in drug design strategies as the circulatory system is fundamental to deliver drugs into cells around the body. First, the glycocalyx may be a target by itself. Our working hypothesis is that cells use the glycocalyx to sense their environment and adapt to it[1]. We can use this to our advantage to trigger responses in the cells by acting at the outer glycocalyx surface without the need to reach the cell interior, which is far more complicated. In particular, we aim to design potential therapeutics which can modulate the structure of the glycocalyx by direct interaction. Second, the interaction of the drugs and their carriers with the glycocalyx will condition to a large extend the efficiency and selectivity of these therapeutics. Importantly, the highly charged nature of the glycocalyx results in the fact that ions are a fundamental component of this complicated system. Evidence of this is the modulation in the permeation of sodium ions by the glycocalyx[2]. In particular, one of our working hypothesis is that glycocalyx may be highly regulated by calcium specifically in the proximal membrane region. Closely related to this is the fact that glycocalyx may have a deep impact on the plasma membrane interaction with certain charged molecules such as cationic cell-penetrating peptides. All these questions and their implications can only be explored if a faithful model of the glycocalyx, such as that aimed at in this project, is available.

In this work package we also aim to improve the efficiency of fluid treatment specially targeted to reduce cancer metastasis[3]. For this we attempt to find mechanisms and therapeutics to regulate the density of the glycocalyx[2]. With such tools in hand we can find an optimal compromise to fight cell metastasis, usually enhanced by larger glycocalyx size, without compromising irreversibly the cardiovascular system, which also requires a healthy large glycocalyx. Also, we explore ways to induce environments that reduce cell replication and, therefore, cancer proliferation. For this, we aim to change the way the cell feels the environment by modulating the glycocalyx structure.

Finally, based on understanding of the plasma membrane as an integral partner of the glycocalyx, we aim to address associated molecular processes taking place at the membrane-glycocalyx interface. We will go beyond "classical" protein-ligand interaction as potential therapeutical targets focusing rather on molecular mechanism which potential therapeutics, such as those involving cell-penetrating peptides[4], employ to cross these complex barriers by modifying the membrane structure. We will have at our disposal unique computational and spectroscopic techniques to monitor in great detail such processes with unprecedented temporal and structural resolution, which is essential for such a task.

Building a faithful model of the glycocalyx

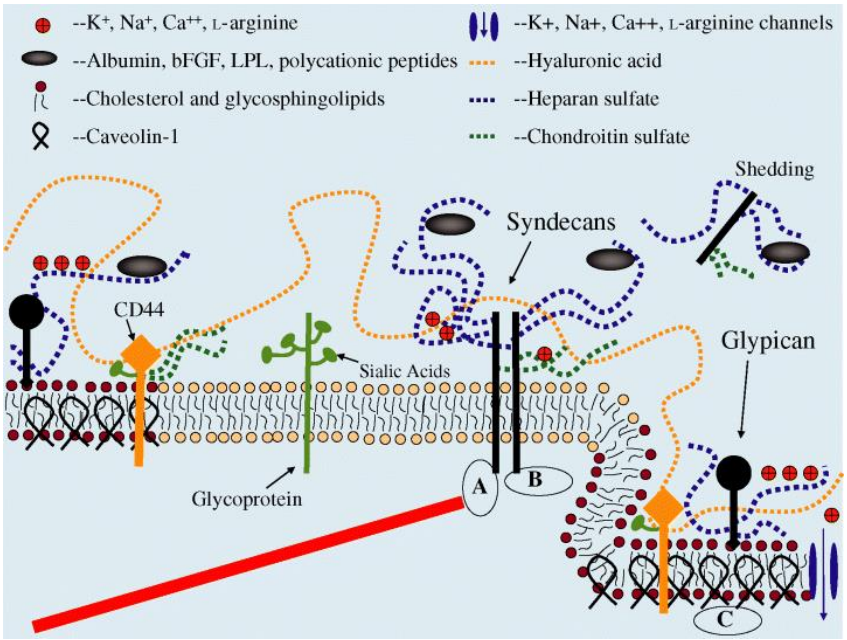

Figure 1 Conceptual model of glycocalyx. Different GAGs types are shown by dotted lines. Syndecan and Glypican are proteoglycans while the rest of the proteins attached to the membrane are glycoproteins. Reproduced from Tarbell et al.1

In this project, the methodological goal of constructing a faithful coarse-grained model of the glycocalyx is aimed at as a first step. This computer model will help us to understand the molecular structure of the glycocalyx and its role as a pharmacological and medical target. This molecular model should provide us with unique information about structurally relevant interactions between the molecular components in the glycocalyx. Glycocalyx is a fibrous 3D network with 10 - 12 nm diameter protein cores attached to the membrane with a characteristic spacing of 20 nm between them and thickness of around 70 nm on top of the cellular membrane[1]. The real challenge is, therefore, to construct a system big enough to contain all the required elements that would allow us to explore the collective behavior of the glycocalyx, see Fig. 1. This will help identify target motives and design strategies, as well as drugs, to alter such structure at will. Initially, particular interest will be directed to interactions between glycosaminoglycans (GAG) and key core proteins such as syndecans, CD44 and albumin which are all essential in the glyccalyx structure. With such a model, we will study how different GAG sequences and entanglements alter the glycocalyx network structure. We will validate the computational results experimentally by working with models of hydrogels containing these glycosaminoglycans. Also, we will address the question whether the underlying membrane changes its properties due to the presence of the glycocalyx. For this computational work, extensive coupling with experimental fluorescence methods will be critical as the effects are typically very local (but may, at the same time, result in significant metabolic changes). We will gradually include additional components of the glycocalyx such as other glycoproteins. Once structural motives which can be targeted will be identified in the calculations, the workload will be shifted towards experiments that will test the discovered target in vivo or in vitro (see Section II.a).

From the technical point of view, it is fortunate that a modern computing facility was opened in the new building of the Institute in 2014. The Institute provides space of 300 m^2 currently equipped with three rows of racks, out of which 60 racks are dedicated to the installation of computer technology. The computer room is efficiently cooled such that the maximum electric power can reach 400 kW. Currently, there is about 400 nodes with over 4000 CPU cores installed, with the setup allowing to roughly triple the computing capacity in the future.

We are thus perfectly prepared to upgrade the computational facility to a new qualitative level for the purpose of the present proposal, allowing high level of parallelization of computer task requiring a low latency network. This will be accomplished by a purchase of a new dedicated computer cluster for multiple users with roughly 160 CPU nodes connected via a fast Infiniband network and a small number of experimental GPU nodes.

On top of using local computational resources, we have been very successful in securing for major calculations broad access to several European mainframe facilities, including the Julich Supercomputer Center (JUROPA), the Berlin-Hannover Center (HLRN), IT4I in Ostrava, and others, and we will continue doing so also within this project.

Concert of molecular simulations and experiments

Several state-of-the-art experimental techniques will be used to validate the computer models generated in this work. We aim to synthesize drugs and fluorescence labels aimed at modulating and exploring the glycocalyx structure and its interactions with drug carrying cell penetrating peptides. We will also profit immensely from several experimental techniques detailed below to bridge results from computer modelling with experiments at biologically relevant time and length scales (not easily accessible by computational models). All this complementary experimental work will be essential to the completion of the proposed objectives.

Synthesis of foldamers bearing non-natural amino acids

It is well known that arginine rich proteins such as albumine are essential for the glycocalyx structure[2]. We suggest that their positive charge is the main reason as they optimally interact with negative glycosaminoglycans. Based on this principle we speculate that arginine rich peptides should play a similar role. Therefore, they could be used as potential drugs to strengthen glycocalyx structure. Also, oligoarginine-containing peptides have been previously shown to penetrate cell membranes and to being able to deliver cargo from the extracellular matrix to the interior of the cell, these peptides have the problem of all other α-amino acid-derived peptides that they are prone to enzymatic digestion and thus their applicability in vivo is limited[5-7]. We plan to synthesize an array of foldamers composed of polyfunctional non-natural amino acids bearing cationic guanidine units and optional free amino functions (Figure 2) and to study their interactions with the hydrogels mimicking the glycocalyx and phospholipid bilayers of cells. The basic monomeric scaffold can be synthesized in a single step from the available materials[8]. Site-specific guanylation and subsequent peptide coupling provide homo-oligomers or mixed oligomers with natural amino acids. The advantage of using the new non-natural oligopeptidic foldamers is that their secondary structure can be independently determined by NMR and/or crystallography, because they are conformationally constrained. Another advantage is that the cationic guanidine units can be attached at varying distance and defined spatial orientation from the backbone of the foldamer backbone. This approach will allow optimizing cell penetration properties or interaction with glycosaminoglycans. Since the non-natural amino acids are polyfunctional they provide at the same time the covalent or multipoint supramolecular binding of cargo, may that be drug-like molecules or reporter units, such as fluorescent molecules to investigate the interaction with the phospholipid bilayers and the penetration process through the membranes. Thus employing our newly developed foldamers of non-natural amino acids containing guanidinium units will shed light on the multiple interactions between interaction of cationic oligopeptides with lipid bilayers and the glycocalyx.

In the newly identified biologically active peptide foldamers we will study their hydrolytical stability in serum and cell extracts. Since they will contain non-natural amino acids and will be conformationally rigid, it is very likely that they will be sufficiently stable toward hydrolysis. However, in cases when the hydrolytical stability will be limited, we will design and synthesize stable non-hydrolyzable peptidomimetics.

The IOCB has opened a brand-new laboratory building purely dedicated to modern organic synthesis in 2014. The applicants group is located in this building. The laboratories are equipped for state of the art organic synthesis, i.e. for the application of Schlenk and vacuum techniques for extensive handling of moisture and air-sensitive organometallic reagents. The group has photochemical equipment for synthesizing strained oligopeptides. Besides its synthetic capabilities, the group has the equipment at

hand to analyze the products and intermediates by HPLC and other techniques. Automated chromatography is available for the purification of the products to be synthesized.

The institute has state of the art analytical facilities for the characterization and structure elucidation of the synthesized compounds. The mandatory and available equipment include 850 and 600 MHz NMR spectroscopy including all advanced techniques necessary for the structure elucidation of the proposed products. Appropriate mass spectrometry techniques, IR spectroscopy, fluorescence spectroscopy and circular dichroism measurements are available as a service at the IOCB. X-Ray analysis is possible in collaboration.

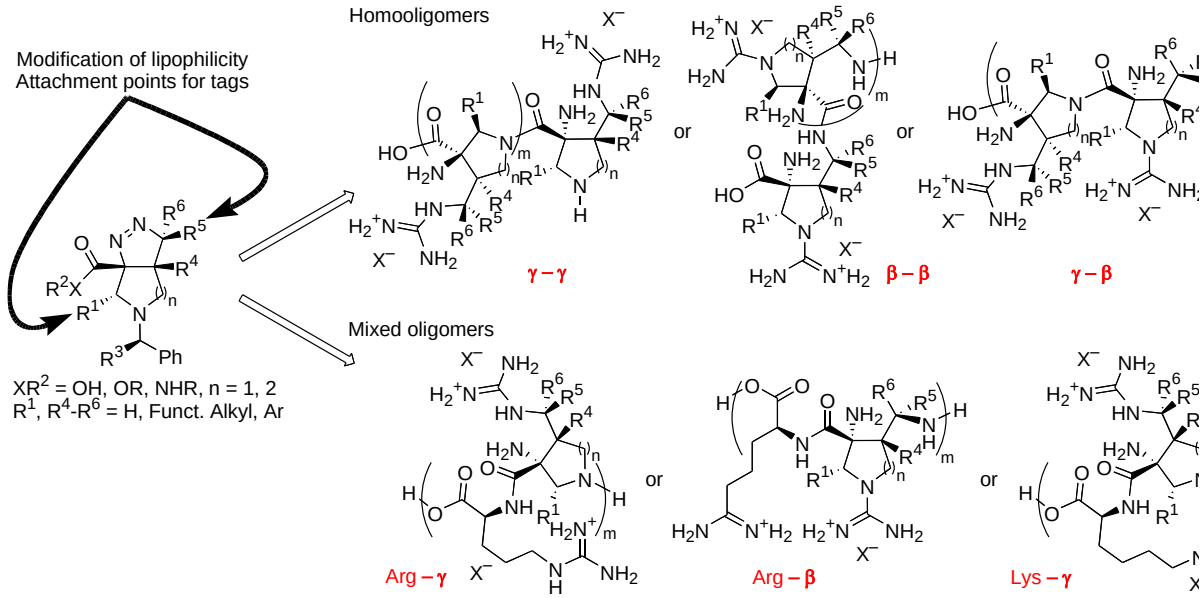

Fig. 2. Examples of cationic oligopeptides to be studied for cell penetration and glycocalyx (de)stabilization.

Two-photon polarization microscopy

Many pharmaceutical agents act by modulating activity of membrane proteins. In order to understand the effects of existing drugs and to find new ones, we need to be able to observe membrane protein function. A new, promising approach to visualizing functions of membrane proteins is two-photon polarization microscopy (2PPM), a technique developed by our laboratory[9-11]. 2PPM works by utilizing anisotropic optical properties of fluorescent molecules in order to obtain information on their orientation with respect to the lipid membrane, see Fig. 3. Thus, 2PPM can report on the existence of distinct lipid phases and microdomains, but also on conformational changes in membrane proteins and changes in membrane-protein interactions. Apart from 2PPM, the Laboratory of Advanced Optical Imaging has at its disposal both the microscopic equipment and the necessary expertise to carry out a wide range of optical microscopy observations. Furthermore, the Laboratory is well equipped for observations of artificial lipid membranes and living cells. To this end we also employ cell biology techniques, *e.g.*, to develop new genetically encoded fluorescence probes.

These techniques will contribute to the project largely by providing experimental information on interactions of fluorescently labelled membrane-penetrating peptides (see Section II.a) using synthetic lipid vesicles. Synthetic lipid vesicles are a convenient model experimental system that allows direct comparison of results of computerized molecular dynamics simulations with experimental observations. Apart from simply establishing the rate of membrane penetration and transport, by using 2PPM we should be able to also gain information on the nature of the interactions between the membrane penetrating peptides and membranes. Apart from observations in model lipid systems, observations will also be made in living cells, extending the findings made in artificial lipid vesicles, and possibly capturing the glycocalyx effect in such process. The development of 2PPM into a high-throughput screening technique should allow rapid screening of peptides for a particular type of membrane interaction and drug discovery.

Apart from observations of membrane penetrating peptides, we plan to make 2PPM investigations of a range of pharmacologically important membrane proteins and associated molecular processes in order to elucidate the molecular mechanisms involved and to aid in development of novel pharmaceutical drugs.

The centrepiece of the laboratory is a new laser scanning two-photon/confocal microscope Olympus FV1200. This microscope is highly versatile and allows the performance of a broad range of microscopy experiments. The microscope has now been adapted for single- and two-photon polarization microscopy, the main focus of the laboratory. Apart from optical microscopy, the laboratory has all equipment and expertise needed for molecular biology, cell biology and basic biochemistry. Several members of the lab are involved in Czech Republic's Research Infrastructure for Systems Biology (C4Sys) project, providing access to a wide range of other resources.

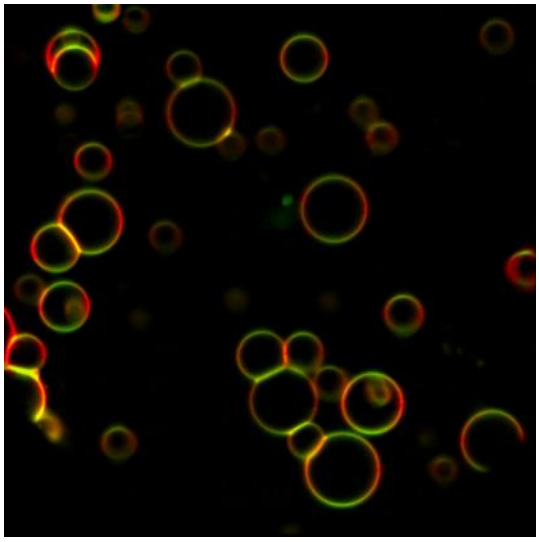

Fig. 3 Giant unilamellar vesicles made of synthetic lipids, containing a fluorescent dye (F2N12S), imaged by two-photon polarization microscopy. Fluorescence excited with light polarized horizontally and vertically colored red and green, respectively. The red/green pattern provides information on orientation of the F2N12S molecules within the lipid bilayer, and therefore on the molecular structure of the lipid membrane9. Similar effects can be seen in living cells7

Advanced spectroscopic techniques for optical activity

Conformational protein behaviour, as in arginine rich peptides, is crucial for penetration of protein-based drugs into the living cell, their stability and biological function. Conformational disorders also cause serious medical conditions, such as protein misfolding diseases, proteinopathies or proteopathies, and malignant cancerous tissues, which are often accompanied by changes in protein structure that can be captured by spectroscopic methods. In solutions only a limited number of methods can be used, however, to study protein conformation. We will therefore use and develop optical spectroscopic methods to track conformation of model systems and verify the computational models. In recent years, such methods are also intensively pursued as "chemical imaging" diagnostic techniques; they are supposed to bring more objectivity and information compared to standard microscopic and histology procedures.[12-13] However, so far such techniques are mostly used on the basis of empirical knowledge. We will, therefore, explore several aspects of the protein folding and interactions and their capturing in optical spectra. Vibrational chiral spectroscopic methods will be primarily used as they are non-invasive, suitable for biological samples, and bring about increased informational content.

Recently, we have shown that spectra of vibrational Raman optical activity (ROA) can not only well-distinguish various protein conformers, but that they can be also simulated with a high accuracy allowing for detailed molecular interpretations (Figure 4).

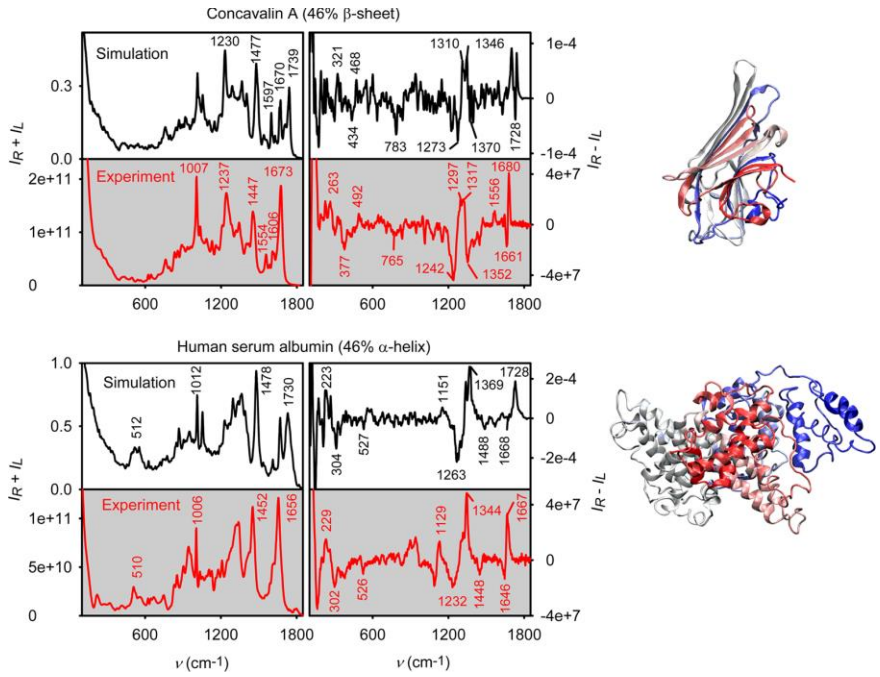

Figure 4. β sheet versus α-helical protein: Simulated and experimental Raman and ROA spectra from our lab.

Unlike vibrational circular dichroism, the ROA spectrum reports both on conformation of the backbone and amino acid side chains. To extend the technique to low physiologic protein/peptide concentrations, we will explore the possibilities of surfaced enhanced Raman scattering in principle able to increase the signal by a few orders of magnitude; preliminary results suggest that the technique is suitable also for the detection of protein ROA.

For separate monitoring of the backbone conformation, we will adapt spectroscopy of the vibrational circular dichroism (VCD). VCD has been standard for protein conformational studies, but also require rather high protein concentrations. Therefore, we will explore the confinement of protein molecules in micelles. This will increase protein solubility, well-mimic the cellular membrane, and enable to detect the interaction with surfaces/heterogeneous environment. Changes in the protein backbone conformation induced by the protein-membrane interaction are directly observable as a shape change of the "amide I" vibrational band and, therefore, show great potential to follow peptides as they penetrate the membrane or interact with the glycocalyx. The extension of ROA and VCD techniques to model protein receptors, such as those based on glycoproteins, will enable the detection of protein binding to the most probable glycocalyx and membrane receptors.

Most spectroscopic structural studies will be performed at the Institute which has established CD (Jobin Ivonne) and ROA (equipped with the BioTools spectrometer) laboratories, and using a recently purchased VCD spectrometer (Biotools). However, an HPLC chromatograph is needed and planned as an investment to guarantee sample purity of model and synthesized compounds. The temperature cell for VCD will be constructed in the Institute workshop enabling the acquisition of thermodynamic protein data.

Crossing complex membrane structures with metabolic implications

We aim to use the above computer modeling and experimental techniques to provide eventually a complete understanding of the strategies adopted by different molecules which are known to make their way to the membrane surface and across it. As a prominent example, we demonstrate in more detail below how we will study with molecular resolution the mechanisms used by cell-penetrating peptides rich in arginines. This is of particular interest since electrostatic interactions between the negatively charged components of the glycocalyx and the positively charged arginine residues of proteins such as albumin (which is an integral part of glycocalyx) are known to stabilize the glycocalyx structure (which becomes disrupted when protein is removed leading to its collapse)[2].

Arginine-rich cell penetrating peptides have recently drawn considerable attention due to their ability to readily cross phospholipid membranes and deliver various molecular cargos inside the cell, including nucleic acids, proteins, quantum dots, and various drugs[14]. It emerges from these studies that one of the structural requirements for the effective penetration of a peptide through the phospholipid bilayer is the presence of multiple guanidinium cations found in arginine residues. Experimental results obtained by confocal microscopy and flow cytometry have shown that oligoarginines containing six or more amino acids internalize more efficiently than equally long lysine oligomers. The exact mechanism of peptide penetration is poorly understood and various mechanistic explanations have been proposed such as inverse micelle formation, electroporation, endocytosis and anion mediated energy-independent diffusion through the membrane. However, none of the proposed mechanisms is able to predict the difference in membrane permeability of arginine vs. lysine containing peptides since they do not take into account the molecular specificity of the residues.

We will aim at clarifying the molecular origin of the different membrane permeability of cationic peptides of varying length and composition. It has been proposed that guanidinium cationic groups present in arginine containing peptides possess the ability to pair via like-charge ion pairing. In contrast, ammonium cationic groups found in lysine containing peptides do not show any sign of stabilizing like-charge ion interaction. This unusual property of guanidinium cations is one of the driving forces for oligoarginine aggregation in water. Oligoarginines also tend to make aggregates at the lipid bilayers as revealed by our pilot all-atom molecular dynamics simulations[14]. Using molecular simulations, we will address the question whether the higher charge density of membrane adsorbed oligoarginine aggregates (in comparison to single oligoarginines or oligolysines) plays a key role in their varying membrane permeabilities. Based on our previous investigations of differences between arginine-rich and lysine-rich peptides, we will aim at unravelling the molecular origin of the role of cationic specificity in membrane permeability. In other words – what is so special about arginine-rich peptides in their ability to penetrate into membranes and how could we exploit it in delivering potential therapeutics into the cell? Modulating cell penetrating peptides and tuning them so as to act selectively for particular membrane structures and compositions in the proximal region of the glycocalyx thus represents a promising, hitherto undruggable target.

Implications for cancer treatment and cell proliferation

When considering cancer and cell proliferation, we will focus our attention mostly on the bulk region of the glycocalyx. This region is rich in glycosaminoglycans, albumin, and other polyfunctional glycoproteins. Strengthening the glycocalyx has been recently proposed to be beneficial to reduce the cardiovascular risk, since the glycocalyx provides a protective layer in endothelial cells, thus limiting infiltration of inflammatory mediators. The opposite turns out to be true in the development of cancer metastasis. Here, the degradation of the glycocalyx reduces the rate of cell division and the onset of cell migration. Albumin, a protein rich in arginine, is an essential structural component of the glycocalyx. In fact, it is one of the few components clinically usually used to strengthen the endothelial glycocalyx.[2] It is believed to act as a crosslinker for the complex glycocalyx network, especially glycosaminoglycans.

In this work package, we want to explore the interaction of albumin with glycosaminoglycans using computational methods and in vitro experiments using hydrogels containing glycosaminoglycans. From those experiments, we expect to learn the main features which make albumin an important crosslinker, which is likely due to its positively charged guanidinium side chains. Another intimately connected and underappreciated target is the interaction of polycationic peptides with the glycocalyx. The interaction of oligopeptides with membrane proteins and the glycocalyx may either stabilize or destabilize it in a similar way as albumin does. The layer of polymeric carbohydrates may also interact in multiple ways especially with cationic oligopeptides and, in this way, either stabilize or destabilize the glycocalyx.

For the above studies, especially oligopeptides, which do not easily penetrate through the lipid membrane, will be valuable. Thus, we aim to employ molecular dynamics simulations in combination with in vitro experiments to influence the structure of the glycocalyx using such oligomers. The experiments will be done in hydrogels containing glycosaminoglycans by studding their rheological properties upon the addition of foldamers of non-natural amino acids containing guanidinium units synthesized for this purpose, which are not as easy to degrade *in vivo* as peptides containing natural

amino acids. In this way, we aim to develop tools to influence the structure of the glycocalyx and to understand how to optimally influence the glycocalyx structure in the context of cancer metastasis, while not compromising the vascular system.

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Research schedule

2018

Targeting glycocalyx structure:

- Development of coarse-grained models for the main structural motives in the glycocalyx bulk region, *i.e.* glycosaminoglycan–glycosaminoglycan and glycosaminoglycan–albumin interaction.
- Development of hydrogels containing glycosaminoglycans as *in vitro* glycocalyx models.
- Calibration of 2PPM assay to explore relevant interactions in the glycocalyx such as the albumin–glycosaminoglycans interactions.

Targeting the cell plasma membrane and its interior:

- Study of the interaction of arginine rich peptides with model plasma membranes using molecular dynamics atomistic and coarse-grained resolution.
- Synthesis and characterization of foldamers constituted by non-natural amino acids containing guanidinium units and other functional groups.
- Synthesis of fluorescent probes of peptide/protein structure.

2019

Targeting glycocalyx structure:

- Development a fully functional model of the glycocalyx bulk region to be use in coarse-grained molecular dynamics simulation.
- Development of a coarse-grained models for the main structural motives of the glycocalyx membrane proximal region, *i.e.* CD44, Syndecan.
- Characterization of the hydrogels models of the glycocalyx upon the addition of foldamers specially designed to perturb the glucocalyx structure.

Targeting the cell plasma membrane and its interior:

- Study of the interaction of the arginine rich peptides with more complex membranes, e.g. membranes containing proteins.
- Performing ROA and VCD experiments with micelles and heterogeneous systems, aimed at elucidating the action of the self-penetration peptides action
- Using of 2PPM to monitor cell penetration by cationic peptides.

2020 -2022

Targeting glycocalyx structure:

- Development of a full coarse-grained model of the glycocalyx including bulk and membrane proximal region which should capture its main features.
- Usage of the model to explore possible targets to change its structure effectively.
- Application of the glycocalyx model to study its repercussion in drug delivery systems.
- Extension of the spectroscopic techniques such as ROA and VCD to the glycoprotein systems.
- Confrontation of computational prediction of protein dynamics in a heterogeneous environment with experimental results

Targeting the cell plasma membrane and its interior:

- Usage of arginine rich peptides and foldamers as drug delivery methods. We will explore this issue *in silico* and *in vitro*. The collection of spectroscopic techniques we aim to develop will be intensively use during this part of the project.
- Developing computational protocols allowing to account for the dynamics and environment in vibrational molecular spectra to study details of the peptides membrane interaction.

Publications and patents

Publications

	Jimp
2017	0
2018	3
2019	4
2020	4
2021	4
2022	5
Total	20

Journal of the American Chemical Society
Journal of Physical Chemistry Letters
Journal of Physical Chemistry B
BBA Biomembranes
Biophysical Journal
Physical Chemistry Chemical Physics
Journal of Chemical Physics

Cooperation with foreign institutions

- Laboratory of Biointerfaces at the Pennsylvania State University: spectroscopic and thermodynamics measurements of affinities of charged peptides to complex biological membrane structures.

- Institute Laue-Langevin in Grenoble: characterization of interactions between biologically relevant charged species in water via neutron scattering