Multiscale Allostery: Basic Mechanisms and Versatility in Diagnostics and Drug Design

There were several significant milestones in the last century, from the discovery of the Bohr effect[1] to understanding that every dynamic protein is intrinsically allosteric.[2–3] It started slowly and took several, decades-long steps to make progress in the analysis of oxygen binding cooperativity[4–5] before the term allostery was coined in 1961.[6] The seminal Monod–Changeux–Jacob work "Allosteric proteins and cellular control systems"[7] provided the first formal description of allostery, followed by its implementation in the phenomenological Monod–Wyman–Changeux[8] and Koshland–Nemethy–Filmer[9] models developed for oligomeric proteins. About a decade later, the key role of protein dynamics[10] was formalized in the free-energy landscape view,[11–12] which allows to uniformly describe allosteric regulation of protein function[13] in the context of conformational ensembles with transitions between corresponding functional states.[3,14] On the brink of 21st century critical improvements in resolution of major biophysical techniques, such as X-ray crystallography and NMR, turning them, at the same time, into high-throughput approaches delivered atomic details and pictures of dynamics for tens of thousands of proteins and protein assemblies.[15] These data revealed the pervasiveness of allostery[2] in a wide spectrum of proteins from small single-chain structures to huge molecular assemblies, machines and beyond,[16–17] showing how allostery affects multiple structural and functional facets of biomolecules on different scales. As a result, the number of works on the physical bases, molecular mechanisms, and biological implications of allostery rapidly increased,[18] expanding the "allosteric territory" borders[19] to include the realm of engineering and design with multiple biomedical applications.[15] In particular, allostery became one of the cornerstones of the precision medicine paradigm,[18] providing a foundation for development of personalized therapies with distinct specificity and dosage. A possibility to circumvent the drug toxicity and emerging resistance, non-competitive and highly specific mode of action are major advantages of prospected allosteric drugs.[20–22] Growing evidence of the contribution of allosteric mutations to expansion of the cancer landscape[18,23] and its involvement in other pathological developments call for considering them in molecular diagnostics and therapeutic strategies.[18,20,24]

We present here a collection of reviews and original research works covering different aspects of allostery and experimental and computational approaches for their analysis, engineering, and design. Starting from the discussion of allosteric mechanisms elucidated with the help of different experimental approaches, we move on to overview recent developments in theoretical models, computational protocols, and results of their applications combined in some cases with experimental approaches. Next, we discuss biomedical implications of allostery, emphasizing the role of allosteric mutations and design of prospected allosteric drugs. The design/engineering theme is continued by considering a relatively new topic – design of allosteric switches and potentially a wide range of their applications. The signalling function of allostery on the cellular lever[25–27] is also exemplified here, pointing to yet another direction of a wide and diverse involvement of allosteric mechanisms in intra- and intercellular signalling, which is anticipated to be an active area of future research.[28–29]

Experimental studies, in some cases supported by simulations and modelling, always provide the most illuminating manifestations of different aspects of allostery in a variety of systems. *Triveri et al.*[30] demonstrated advantages provided by the allosteric mode of regulation in the case of protein isoforms with conserved active sites but distinct cellular localizations and functions. They considered TRAP1, the mitochondrial paralog of Hsp90 family of molecular chaperones, which is a key component in the cell metabolic machinery and an important player in development of pathological conditions. Leveraging on the previously designed lead compound for TRAP1 inhibition, the authors used computational analysis for design of optimized effector derivatives, efficacy of which they experimentally validated in biochemical and cellular tests of the chaperones ATPase activity. Based on this case study of allosteric inhibition of TRAP1 ATPase upon unscathed Hsp90 activity, the authors propose to generalize their approach for getting new derivatives of allosteric effectors with better drug-like profiles and improved biochemical and cellular activities. The challenging task to

understand the mechanism of allosteric signalling associated with protein–protein interactions was tackled by *Heckmeier et al.*.[31] They used transient infrared spectroscopy to explore the allosteric signal propagation in the myeloid cell leukemia 1 (MCL-1) protein caused by the binding of its natural moderator - pro-apoptotic BIM peptide. In order to study subtle conformational changes associated with MCL-1/BH3-only complex formation, the azobenzene switch was cross-linked to the BH3-only (here, BIM) peptide in different sites. The light induction of the switch selectively disturbs the structure, with distinct molecular response detectable via transient IR spectroscopy. The authors obtained a comprehensive picture of dynamics spanning the time range from pico- to microseconds and revealing the sequence of events starting from the photo-induced perturbation of the BIM peptide to propagation of an allosteric signal in the MCL-1/BIM complex. NMR-based studies were proven to be instrumental in the analysis of protein dynamics and function-related conformational changes. Here, combining solution NMR spectroscopy with molecular dynamics (MD) simulations, *Maschietto et al.*[32] mechanistically characterized the enhancement of *Mycobacterium tuberculosis* tyrosine phosphatase (MPtpA) activity driven by the allosterically acting mutation. They showed that mere removal of polar side chain Q75L leads to substantial changes in the catalytic site, describing the potential mechanism of MPtpA activation as part of the *M. tuberculosis* pathogenic life cycle. In another work, *Winston et al.*[33] used NMR relaxation dispersion experiments to understand the conformational fluctuations on the microsecond-to-millisecond timescale that occur in chorismate mutase from *Saccharomyces cerevisiae* (ScCM). They found indications of a potentially-three-state system, contrary to an earlier proposed two-state model of fluctuations between activated and inhibited states in the absence of effectors. Additionally, they detected negative cooperativity, leading to different catalytic activities of single and double allosteric effector-bound states. The authors also hypothesized an involvement of ScCM in a variety of stress responses, linking it to their earlier observation of positive cooperativity of Trp binding at lower temperatures.

Wealth of experimental data delivered by different biochemical and biophysical methods and high-throughput proteomic approaches provided a solid foundation for development of theoretical and computational models. *Arantes et al.*[34] reviewed here computational approaches for elucidating allosteric regulation in protein-nucleic acid complexes. They showed how accelerated MD simulations can be used to construct "enhanced network models" that describe the allosteric response over long timescales. Combining graph theory with *ab-initio* MD and quantum mechanics/molecular mechanics (QM/MM) simulations allows to follow step-by-step dynamics and allosteric regulation of catalytic function. Three archetypes of allostery in protein-nucleic acid complexes - the nucleosome core particle, the CRISPR-Cas9 genome editing system, and the spliceosome - are showcased in this review. *Dubanevics and McLeish*[35] challenged themselves with a task of optimising Elastic Network Models for studies of protein dynamics and allostery. They considered four parameters, including distance and number of modes cutoffs, special parametrization for the covalently connected backbone, and effects of different ligand representations. Studying three relatively small homodimeric proteins, they came up with a set of recommendations for setting simulation parameters. Their conclusion on the relationship between structural type and the cutoff for the number of considered modes calls for additional studies, especially in the case of high-throughput simulations of protein dynamics using the coarse-grained models. The robustness of other discussed parameters should be further confirmed for a wide diversity of structures with different sizes and architectures. *Vargas-Rosales and Caflisch*[36] proposed an original approach for the analysis of allosteric regulation using molecular dynamics (MD) simulations. They performed unsupervised analysis of MD trajectories, projecting the free energy along the slowest relaxation eigenvector. Using the case study of peptide binding to the PDZ domain, the authors showed that their approach allows to enhance sampling of simulations and to obtain residue-level reconstruction of the structure. *Altintel et al.*[37] developed a new computational approach, assuming that allosteric communication can be treated as an information transfer between functional and regulatory sites. They used a transfer entropy for obtaining the picture of information flow between the residues/sites, which, in turn, are determined by considering the degree of collectivity in the information transfer. The authors concluded that high collective information characteristic for functional/regulatory sites may be the result of the protein topology optimisation, understanding of which can be instrumental in engineering and design efforts. Similar conclusions are presented in[38] published in this issue, where the evolution of protein folds is shown to be an important determinant of the fold-dependent allosteric control and its diversification with implications for design of biologics and allosteric effectors. Further developing the bond-to-bond propensity analysis built upon energy-weighted atomistic protein graphs, *Wu et al.*[39] proposed here a method for computing and scoring paths of optimised propensity that link the orthosteric site with the identified allosteric sites and for identifying crucial residues that contribute to those paths. Illustrating their method with example of three proteins, hRas, caspase-1, and PDK1, they show that it allows to identify key residues in both orthosteric and allosteric

sites, as well as the pathway connecting them and providing, according to the authors, alternative targets for drug design. It will be interesting to see further support for the proposed role of the connecting paths, which would show that they can be targeted in drug development efforts rather than serve as a mere indicator of conformational changes initiated by corresponding allosteric signalling. In another work, *Stromish et al.*,[40] same group of researchers using bond-to-bond propensity in combination with Markov transient analysis – both fully atomistic graph-theoretical methods – performed an analysis of the SARS-CoV-2 main protease. Performing statistical bootstrapping for scoring predictions, they identified four putative allosteric sites as candidates for allosteric drugs. On the basis of predictions and experimental verifications of allosteric sites in the Src kinase, *Mingione et al.*[41] developed a generalized computational protocol for predicting allosteric sites on the basis of unbiased ligand binding simulations. The authors claim that experimental assays confirming the allosteric nature of the predicted binding site presented in the work provide a proof-of-concept for applying their protocol to other protein–ligand systems. *Tee et al.*[38] combined an analytical and predictive power of the structure-based statistical mechanical model of allostery developed in the group (SBSMMA[42–44]) with understanding of the polymer nature,[45] hierarchical structure,[46] and evolution[47] of proteins for exploring the conservation and diversity in allosteric signalling with implications for engineering and design.[48–50] They proposed that pictures of allosteric signalling observed in different fold types, multi-domain structures, and oligomeric assemblies can be used in engineering and *de novo* design of proteins with allosterically regulated functions. *Wingert et al.*[51] investigated the signature dynamics of Class A GPCRs – rhodopsin-like GPCRs, showing the way to link specifics of structural dynamics to mechanisms of activation, subfamily differentiation, and speciation. The distinct role of different components of intrinsic dynamics was also demonstrated: global, the most cooperative motions provide the signature dynamics of the family, while high-frequency, local motions determine subfamily-specific features. The variance in the dynamics of global modes was shown, in turn, to be related to GPCR activation. *Post et al*[52] elucidated the mechanism of the global open-closed motion of two domains of T4 lysozyme involved in its allosteric transition. Obtaining microscopic details of the time evolution of conformational transitions associated with dynamical mechanism of allostery, the authors determined essential internal coordinates and corresponding energy landscape for characterizing the transition path. They observed cooperativity of global open-closed transitions, which emerge from local fluctuations and short-distance couplings upon the perturbation caused by the (un)binding of a ligand. Pointing to the analogy with the explanation of cooperativity of large-scale transition via interaction of local fluctuations provided by Ising model, the authors plan to use it in their future work. *Khamina et al.*[53] proposed a critical perspective on the origins and mechanisms of allostery in cyclic nucleotide dependent kinases. They discussed a wide range of allosteric effectors, including non-cyclic nucleotides, metals, disease-related mutations (DRMs), and post-translational modifications in the form of disulfide bridges. In addition to rather general mechanisms of allostery, such as a shift in dynamical equilibrium and modifications of intramolecular interactions, the authors hypothesized a possibility for allosterically triggered oligomerization and/or phase-transition caused by DRMs originating transient unfolding and exposure of amyloidogenic sites. *Celebi & Akten*[54] used molecular dynamics simulations for exploring two allosteric sites in *S. aureus* phosphofructokinase (SaPFK). They showed molecular details of signalling that provide potentially opposite modes of regulation upon binding to these sites. *Verkhivker*[55] performed a computational analysis of Hsp90-Hsp-70-Hop-CR complex examining allosteric mechanisms of Hsp90 chaperone interactions and chaperone-dependent client recognition and remodelling. *Lu et al.*[56] employed multi-replica Gaussian accelerated molecular dynamics (GaMD) simulations for investigation of long-range allosteric communication in theSPRED-Ras-NF1 complex involved in regulation of the Ras GTPase cycle.

Several analytical frameworks and computational protocols for engineering and design of allosteric effectors and biologics were also recently developed. *Zha et al.*[57] presented the Allosteric Database (ASD), describing four major categories of data: structure of documented allosteric proteins and sites, genetic information, allosteric modulators, and allosteric mechanisms. Different applications on the basis of the above data, including studies of allosteric mechanisms, allosteric site detection, effects of mutations, and design of allosteric modulators, are also discussed. Combining molecular dynamics simulations with dynamic residue network analysis, *Tastan Bishop et al.*[58] proposed a computational protocol for allosteric drug discovery enabling analysis of allosteric effects of mutations, prediction of potential allosteric sites, and identification of allosteric modulators. *Li et al.*[59] raised a question whether covalent allosteric effectors targeting cysteine residues in low-affinity binding pockets in Ras and kinases can be developed. They examined known allosteric effectors and sites in these proteins, obtained their AlphaFold models and conducted 3D search of pockets to find cysteines in the proximity of known structurally characterized ligands that bind the pocket. The authors proposed that prospected covalent allosteric chemistry can be beneficial for converting suboptimal binding pockets into druggable ones, facilitating selectivity and efficacy, and helping to overcome

resistance mutations. Using examples of Class A GPCRs and CMGC protein kinases, *Tan et al.*[60] explained how Allosteric Signalling and Probing Fingerprints obtained on the basis of SBSMMA[42–44] can help to predict allosteric sites and to design ligands for them. Evaluating differences between allosteric and orthosteric sites and ligands, they argued that rational design of allosteric drugs should include a mutual adjustment of the site-ligand pairs in order to originate desired allosteric signalling, and proposed a generic protocol for computational design of allosteric effectors and for allosteric tuning of biologics.

A wide spectrum of increasingly important tasks in the intersection of precision medicine and allostery presented here in several reviews and original research works. *Nussinov et al.*[24] considered two opposite poles of allostery in the realm of biomedical implications: allosteric cancer drivers and allosteric drugs. The effects of cancer drivers are exemplified here by the case studies of $PI3K_a$, Raf kinases, and PTEN tumor suppressor phosphatase. Discussion of allosteric drugs is focused around two innovative examples: (i) molecular glues that act as non-competitive active site stabilizers or inducers of protein–protein interactions and (ii) PROteolysis TArgeting Chimeras (PROTACs) – heterobifunctional degraders that bind to a target via allosteric inhibitor, which is, in turn, linked to the warhead molecule delivering the ubiquitination complex to the target domain and securing degradation of the latter. *Qiu et al.*[61] showed that allostery provides an opportunity to drug a difficult target, a key regulator of epoxy fatty acid (EpFA) metabolism – human soluble epoxide hydrolase (hsEH). Building on their previous success in allosteric inhibition of hsEH and using a combined biochemical, biophysical, and computational screening, the authors explored the properties and behaviour of three electrophilic lipids belonging to the class of the nitro fatty acids, 9- and 10-nitrooleate and 10-nitrolinoleate. They found that nitro fatty acids can covalently bind to two allosteric sites newly predicted here,[62] pointing to a possibility of future fragment-based design of allosteric effectors for hsEH with increased efficacy and selectivity. Using a combination of structure-based computational approaches *Deng et al.*[63] shed light on the complexity behind actions of cancer drivers. Studying 1132 variants in the p53 DNA-binding domain (DBD), they identified a group of mutations having a marginal effect on structural stability, but allosterically affecting the interface involved in p53 functional protein–protein interactions. Further validating their predictions with the help of enhanced sampling methods for 15 variants, the authors suggested that techniques used in this study can be applied more broadly to other proteins, helping to prioritize variants for experimental validation and to identify isoforms alterations on the way to personalized therapies. An interesting example of specificity in the effect of mutations in oncogenesis is considered by *Grudzien et al.*.[64] Performing comparative computational analysis of the NMR and X-ray crystallographic data they showed that while structurally similar, G12V and G12D mutations of KRas GTPase reveal different dynamics. Moreover, with the G12V mutant visiting the "active-like conformation" in the virtually inhibited GDP-bound state, this observation can potentially explain the aggressiveness and chemotherapy resistance of the G12V variant. Such mutation-type dependence suggests using distinct therapeutic strategies for inhibiting multiple activities of oncogenic KRas driven by specific mutations.

Design of allosteric switches is another new direction with a great promise for developments in precision medicine. *Fauser et al.*[65] surveyed recent advances in engineering allosteric control of protein function, providing new synthetic biology tools for highly specific and precise regulation of biological processes and opening opportunities for development of innovative therapeutic strategies. Among the major platforms and experimental/practical manipulations the authors considered (i) engineering chimeric GPCRs to obtain synthetic receptors with desired signalling input and output; (ii) achieving regulation of different protein classes by linking the allosteric switch module to the target protein; (iii) *de novo* design of allosteric chemogenic and optogenic allosteric switch domains and synthetic proteins. In more technical but important experimental work, *Ayva et al.*[66] explored the ways to optimise design of artificial allosteric systems based on domain insertion. They used their expertise in designing higher order biosensor architectures and a library of calmodulin chimeras with PQQ-glucose dehydrogenase (PQQ-GDH) - electrochemical molecular switches that can be used in bioelectronic and biosensor applications. Analysing the key performance parameters of switches, they found that the dynamic range and response rates are negatively correlated. They proposed to study the dependence of the switch performance on the reporter domain, role of the optimization of linkers between the acceptor and reporter domains, and the fastest activation rate that can be achieved.

Several works point to an involvement of allostery in the regulation of cellular activity and responses to external stresses, and to a potential avenue of using allosteric features of proteins in design of antibody-based vaccines and drugs. A very elegant study by *Tantrimudalige et al.*,[67] showcases work of allostery in the bacterial cell response to hyperosmotic stress. The authors explored regulation of the *C. glutamicum*'s BetP, a member of betaine/choline/carnitine transporters (BCCTs), which is rapidly activated during hyperosmotic stress, providing an uptake of the osmolyte – betaine. Using Hydrogen-Deuterium Exchange Mass Spectrometry (HDXMS), which emerged as

one of the key methods for exploring the time evolution of dynamical systems, they found that increased intracellular K^+ concentration caused by the hyperosmotic stress results in allosterically initiated betaine and Na^+ binding and transport across the cell membrane. The regulation is initiated by specific interactions between N- and C-terminal domains in presence of K^+, which allosterically stabilize two transmembrane helices forming the Na^+/-betaine binding pocket and optimizing some additional interactions for better betaine binding. *Thomas et al.*[68] experimentally studied involvement of allostery in the mechanical attachment of pathogens to host cells. They considered so-called allosteric catch bond mechanism of bacterial cell attachment, using example of the FimH protein from *E. coli,* a model lectin-pillin protein, changing its conformation between inactive 'low-affinity state' (LAS) with interacting domains to active 'high-affinity state' (HAS). Contrary to earlier opinion that catch bond mechanisms work only in fluidic shear-dependent mode of adhesion, the authors showed that fast and sustained allosteric activation of FimH can occur under static conditions. They also described a more complex picture of the FimH activation-deactivation. In the relevant review by the same group (*Sokurenko et al.*[69]), design of neutralizing antibodies against allosteric proteins on the example of FimH is overviewed and discussed. It appears that antibody response against FimH conformers is discrete. In the case of active conformers allosteric activating antibodies are induced, while for inactive ones – neutralizing orthesteric and parasteric (binding next to the ligand) antibodies. The authors also described a novel type of antibody, which recognizes FimH, regardless of the mannose's presence, and binds equally well to both HAS and LAS, blocking their ability to switch to another conformation and leading the authors to coin the term 'dynasteric'.

To conclude, allostery is a complex signalling phenomenon that can be involved in regulation of practically any dynamical system under external perturbations. Most of the works in this collection reflect recent progress in molecular mechanisms of allostery and its biomedical implications. There are still challenges and exciting questions to be addressed, including a need for transferable computational models, increasing their predictive power and calculation speed, as well as bridging the resolution gap between different scales, experimentally and computationally. Experimentally, high-throughput assays, including structure–activity relationship of allosteric drugs should be characterized not only by the binding affinity, but also the allosteric signal that they promote. Consideration of cellular signal transduction will lead to a multiscale picture of interactions spanning from interatomic interactions in individual molecules to communication in metabolic pathways and intracellular signalling between cellular components.

Acknowledgements

This project (RN) has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261201500003I. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. This Research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. Financial support provided by the Biomedical Research Council via Agency for Science, Technology, and Research (A*STAR) is greatly appreciated (INB).

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