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Diverse Substrate Scaffolds by Multicomponent Reactions and Protein Structural Dynamics by Single-molecule FRET

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Diverse Substrate Scaffolds
by **Multicomponent Reactions**
and Protein Structural Dynamics
by **Single-molecule FRET**

Ruixue Xu

The work described in this thesis was performed at the Department of Drug Design, Faculty of Science and Engineering of the University of Groningen, the Netherlands, and at the Rega Institute of KU Leuven, Belgium. R. Xu was financially supported by the China Scholarship Council (CSC).

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KU LEUVEN

Diverse Substrate Scaffolds by Multicomponent Reactions and Protein Structural Dynamics by Single-molecule FRET

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the decision by the College of Deans

and

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and in accordance with
the decision by the Doctoral School Committee.

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This thesis will be defended in public on

Tuesday 1 February 2022 at 12.45 hours

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TO

MY FAMILY AND FRIENDS

WHO BRING ALL THE PLEASANT MOMENTS TO MY LIFE

PARANYMPHS

Xin Li

Bidong Zhang

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Aim and Scope of the Thesis

AIM AND SCOPE

Part I: Diverse substrate scaffolds by multicomponent reactions

Multicomponent reactions (MCRs) are reactions that have at least three starting materials to form one single product, for example, 3, 4, ... 8, where the majority of the atoms from starting materials can be transferred to final products (**Fig. 1**). [1] MCRs have many advantages: a.) they are highly efficient due to one-pot strategy instead of multiple sequential steps; b.) high atom economics, most of the atoms from starting materials are incorporated in the final products; c.) they are large diversity. [2] Therefore, MCRs are a unique tool that provides a great opportunity to generate large and diverse libraries of molecules in a highly efficient, low-cost, and environment-friendly manner.

Figure 1. The illustration of traditional sequential synthesis (A) and multicomponent reactions (B).

Isocyanide-based multicomponent reaction (IMCR) is one of the most well-studied and extensively explored MCRs. [3, 4] The first isocyanide-based MCR was described by Mario Passerini in 1921 in Florence, Italy, which is known as the Passerini three-component reaction (P-3CR). [5] Hundred of IMCRs have been described over the years, such as Groebke-Blackburn-Bienaymé (GBB) reaction [6], Ugi reaction, and Ugi tetrazole reaction [7] (**Fig. 2**). With the emergence of high-throughput screening in the pharmaceutical industry in recent decades, the discovery of novel MCRs has naturally gotten more and more attention from academic and industrial researchers due to its inherent high efficiency and high exploratory power. [8]

High- and higher-throughput methods have been performed to speed up development and improve the success probability of the research. [9] In the era of machines and automation, synthetic chemistry gets a new lease of life. The construction of the robotic

Figure 2. Representative multicomponent reactions.

system performs tasks of synthetic chemistry in ways that are fast, accurate, and consistent. By coupling those constructions with in-line analytical capabilities, the automated optimization of reaction conditions could be achieved. [10, 11] As an example, continuous flow systems have already been applied to prepare fine chemicals, such as natural products or pharmaceuticals. [12] Although extending the chemical space of well-studied reactions is straightforward, exploring new reactivity and scaffolds keeps challenging.

The first two chapters of this thesis describe the advantage of the combined MCRs and automatic synthesis in generating novel scaffolds to accelerate drug discovery. In **chapter 1**, we used for the first time acoustic droplet ejection (ADE) technology and fast quality control to screen the efficiency of synthetic reactions on a nanomole scale in an automated and miniaturized fashion. The interrupted Fischer indole combined with Ugi-type reactions yielded several attractive drug-like scaffolds. In 384-well plates, a diverse set of interrupted Fischer indole intermediates were produced and reacted with the tricyclic hydantoin backbone in a 2-step sequence. Similarly, preformed Fischer indole intermediates were used to produce diverse sets of Ugi products, and the efficiency was compared with that of the in situ method. Multiple reactions were performed again on a preparative millimole scale, showing scalability from nano to mg and thus synthetic utility. An unprecedented large number of building blocks were used

for fast scope and limitation studies (68 isocyanides, 72 carboxylic acids). Miniaturization and analysis of the generated big synthesis data allowed deeper exploration of the chemical space. They permitted the gain of knowledge that was previously impractical or impossible, such as the rapid survey of reactions and building block and functional group compatibility.

Hit finding in early drug discovery is often based on high throughput screening (HTS) of existing and historical compound libraries, which can limit chemical diversity, is time-consuming and costly. On-the-fly compound synthesis and in situ screening in a highly miniaturized and automated format is a novel and alternative approach. In **chapter 2**, we used acoustic dispensing technology to synthesize a library of 1536 compounds based on the Groebcke-Blackburn-Bienaymé reaction (GBB-3CR) on a nanolitre scale. The library was screened by differential scanning fluorimetry (DSF) and cross-validated using microscale thermophoresis (MST) against the oncogenic protein-protein interaction menin-MLL. Several GBB reaction products were found as μM inhibitors, and the structural basis of the interactions with menin was elucidated by cocrystal structure analysis. Miniaturization and automation of the organic synthesis and screening process can lead to an acceleration in the early drug discovery process and is an alternative to classical HTS.

In **chapter 3**, the fast and straightforward synthesis of polycyclic quinazolinone derivatives is provided. Polycyclic quinazolinones are among the most important class of biologically active N-fused heterocyclic scaffolds that have been involved in many natural products and pharmaceuticals. [13,14] The reaction sequence starts from an Ugi-four components reaction between the commercially available carboxylic acids, aldehydes, isocyanides, and ammonia, and the formed Ugi adducts further undergo a Pd-catalyzed cyclization to create the desired products. The method provides fully substituted polycyclic quinazolinones in two steps, with a total yield of up to 69%.

Part II: Protein structural dynamics by mainly single-molecule FRET

Over the past decades, biophysical technologies such as X-ray crystallography, isothermal titration calorimetry (ITC), microscale thermophoresis (MST), single-molecule Förster Resonance Energy Transfer (smFRET) [15], and hydrogen-deuterium exchange mass spectrometry (HDX-MS) [16] have become important tools for the studying of protein conformational dynamics or protein iso-energetic states which are essential for protein functional study or in drug discovery research (**Fig. 3**). [17]

Figure 3. Typical data were obtained from representative biophysical techniques. (A) An electron density map derived from X-ray diffraction data. (B) A thermogram showed heat absorption rates upon sequential ligand titrations to a protein solution. (C) Fluorescence monitoring of the thermophoretic motion of a ligand. (D) Apparent FERT value varies, demonstrating protein conformational change upon ligand binding. (E) The deuteration level for each peptide is compared at different time points for various conditions.

Single-molecule FRET describes a distance-related and non-radiative energy transfer from a donor to an acceptor on a molecule-by-molecule basis. The basic requirements for the happening of FRET are a). the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor; b). the distance between two fluorophores should be within 2-10 nm; c). the dipole moments of both fluorophores are not perpendicular to each other. This technique allows researchers to look into the conformational dynamics (lifetime: ~picoseconds to ~seconds) beyond the static structures. [15,18] The Protein conformational dynamics dramatically influence the affinity of a drug for its clefts in two ways [19-22]: a). If binding is achieved via an induced fit or a conformational selection mechanism: the binding affinity depends equally on the conformational free energy (protein flexibility) and the binding free energy (strength of the binding pocket). b). If a protein manifests intrinsic dynamics, its binding clefts will not be stable and fixed over time but continuously “dismantled” and reformed. It is critical that both aspects are determined in every drug development effort. However, in practice, due to technical difficulties, such aspects are rarely considered.

Those novel biophysical tools allow the structural dynamics of proteins, and the regulation of such dynamics by binding partners, to be explored in unprecedented detail. Although this has provided critical insights into protein function, the means by which structural dynamics direct protein evolution remains poorly understood. [23] In **chapter 4**, we investigated how proteins with a bilobed structure, composed of two related domains from the type-II periplasmic binding protein domain family, have undergone divergent evolution leading to adaptation of their structural dynamics for function. We performed structural analysis on ~600 bilobed proteins with a common primordial structural core, which we complemented with biophysical studies to explore the structural dynamics of selected examples by single-molecule Förster resonance energy transfer and Hydrogen-Deuterium exchange mass spectrometry. We show that evolutionary modifications of the structural core, largely at its termini, enable distinct structural dynamics, allowing the diversification of these proteins into transcription factors, enzymes, and extra-cytoplasmic transport-related proteins. Structural embellishments of the core created new interdomain interactions that stabilized structural states, reshaped the active site geometry, and ultimately altered substrate specificity. Our findings reveal an as yet unrecognized mechanism for the emergence of functional promiscuity during long periods of evolution and are applicable to a large number of domain architectures.

In **chapter 5**, we went further to analyze a class B (a structural category of *cherry-core*, CC, **chapter 4**) amino acid-binding protein that possesses an open unliganded state stabilized via the C-terminal helical element. The closed state has a dual functional role: to trap a specific ligand but also to activate transport. Here, we reveal the means by which neutral mutations are combined to plastic modifications to modulate the binding and conformational free energy by multi-tier structural dynamics and by that function and ligand specificity. Our findings have important repercussions not only with respect to protein adaptability and allostery but also with specific fields like drug design that examining only the static states of the target proteins is not sufficient for designing a high-affinity binder in some cases.

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