Section: Research Paper

DEL LINKERS: TRENDS, APPLICATIONS, AND OUTLOOKS

Nicolas Duchemin*[a], Zhaomei Sun[b], and Yun Jin Hu[b]

[a] Dr. N. Duchemin

Pharmaron UK, Ltd. Innovation Park, West Cl, Hertford Rd, Hoddesdon EN11 9FH, UKl:

*Corresponding author

Email: Nicolas.duchemin@pharmaron-uk.com

[b] Zhaomei Sun and Yun Jin. Hu

Pharmaron (Ningbo) Technology Development Co., Ltd.

No. 800 Bin-Hai 4th Road, Hangzhou Bay New Zone, Ningbo, 315336 (P. R. China)

DNA-encoded libraries are collections of small molecules covalently bound to single or double-stranded oligonucleotides, which sequences individually carry encoded information about the identity of the molecule. These oligonucleotide conjugates are assembled using building blocks or cores, and their corresponding encoding DNA tags. Libraries, once synthesized, can be screened towards biological targets of interest, and binders, being physically separated from non-binders, can be identified simple decoding of the unique DNA tags, using PCR amplification and DNA sequencing. Ever since the conceptual inception of DNA-encoded library technology in 1992, [1] and following applicative studies, [2, 3] the field has been rapidly emerging as a powerful pathway to discover valuable chemical matter for drug discovery and probe molecules. Thanks to the efforts of many academics and industrial groups, this technology has been continuously evolving and benefited from the multidimensional advancements: new on-DNA chemical reactions, improved selection methods, increasingly affordable nucleotide sequencing, and ameliorated data analysis approach to reveal the enriched binders. [4, 5] The significance and strong impact of DELT in unveiling relevant hits in an efficient and economical manner was recently witnessed by the expedient access to SARS-CoV-2 M^{pro} inhibitors, [6] and naturally became increasingly significant in drug discovery. These efforts notably contributed to the discovery of two phase 2 clinical candidates GSK2256294 and GSK2982772 from GSK and recently FDA-approved X-165 for Phase I clinical trials. [7-9]

On the small molecule side, it is now established that DELs of superior quality are driven by innovative design, [10-13] diverse chemical space, [14, 15] enhanced druggability, [16] and efficient reaction used in synthesis. [17] On the oligonucleotide side, where lies the vital encoded information, a variety of approaches have been developed and democratized. DNA-recorded libraries, consisting in a combinatorial split-and-pool strategy, are built by alternating ligation of building blocks on one end, and corresponding DNA tags on the other (Figure 1 A.). This particularly straightforward strategies is, although widely used, completed by DNA-directed libraries, in which the encoding tag actually guides the ligation of building blocks to one another (Figure 1 B.), and encoded self-assembling combinatorial (ESAC) libraries, also called dual-pharmacophore libraries, [4] in which single stranded and partially complementary tags encoding for distinct bound compounds can be screened to find binders to several adjacent binding sites on a target of interest (Figure 1 C.). Finally, PNA-encoded libraries can be synthesized on support and used in screening. [18]

Nonetheless, although both sides of the concept seem relatively well-covered, one vital feature of DEL is yet neglected. Indeed, the efficacy of DEL in screening campaign wouldn't have been the one we can now witness if the question of the link between the potentially binding small molecule and its encoding DNA tag hadn't been addressed. DEL linkers have been, since the early days of the

technology, a relatively silent matter, but many groups have taken interest in the development of innovative methodologies to increase the diversity and applicability of these linkers. In this review, we will give an overview of the different approaches developed in the past 30 years and their impact on the use of DEL technology in drug discovery, starting by a rapid assessment of linker's importance and influences, and then moving to non-cleavable and cleavable linkers before moving onto the potential future outlooks.

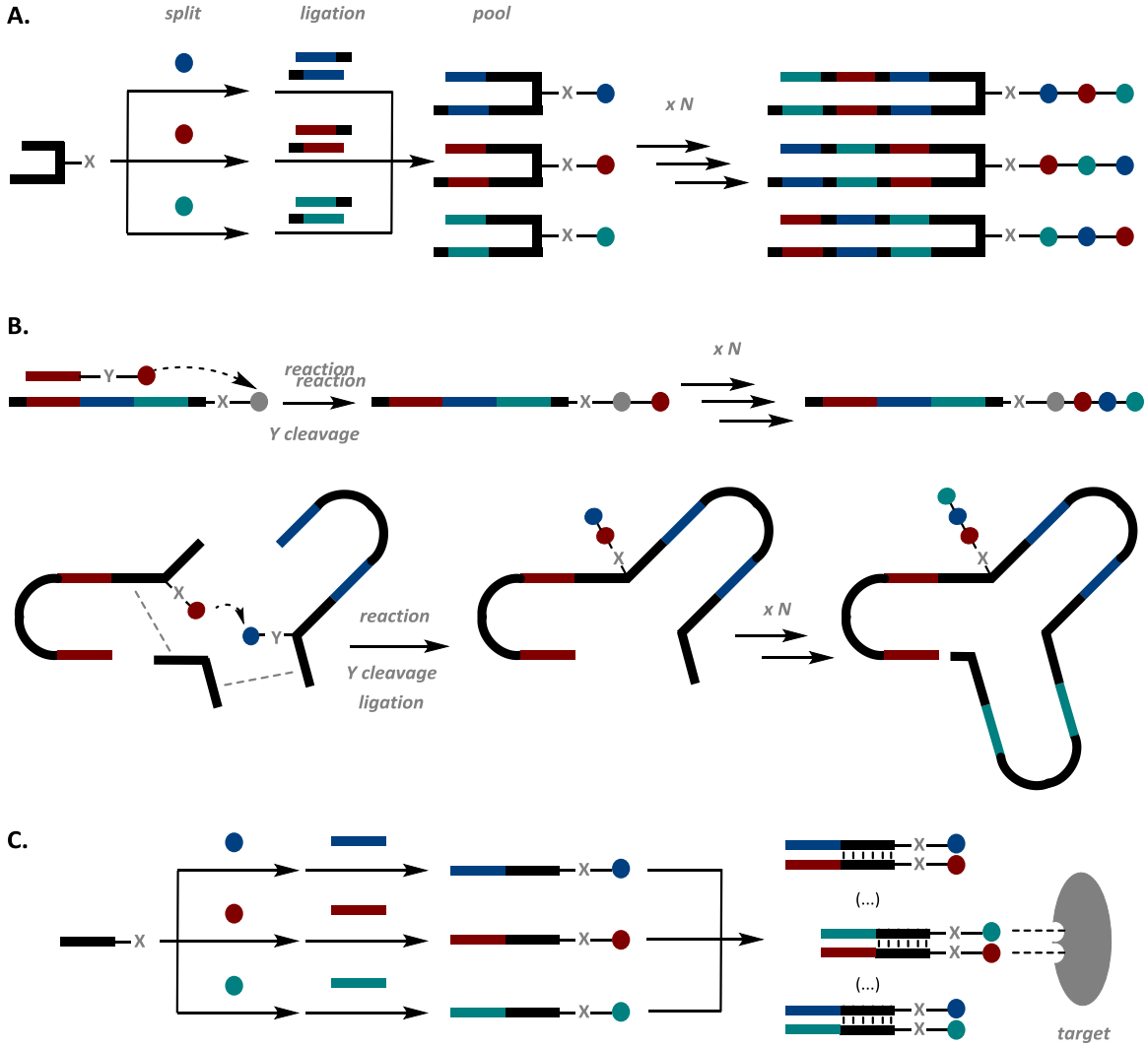

Figure 1. DNA encoding methods. A. DNA-recorded library synthesis (split-and-pool strategy). B. DNA-directed library (using DNA-templated synthesis). C. encoded self-assembling combinatorial libraries (ESAC).

I. DEL linkers: opening remarks

In their seminal publication, Brenner and Lerner described the founding principles of DEL but remained mute on the nature of the chemical moiety linking the mono-, di-, or tri-peptidic assemblies presented and their encoding sequences (Figure 2 A.). The simple but clearly vague term "link" is used to describe this key motif, leaving the field of possibilities wide open, but the following year, Gallop *et al.* [3], and Janda *et al.* put the concept in practice and finally displayed the first drafts of DEL-amenable linkers [2]. However, in comparison with modern practices, these first attempts focused on peptidic libraries, made use of solid-phase synthesis strategy, alternating between oligonucleotide and peptide construction. This natural constraint led to the use of relatively long alkyl chains, linking the support to molecular handle such as a threonine residue, capable of engaging in oligonucleotide synthesis on the hydroxyl moiety, while the amino group could be used for peptidic

synthesis. Interestingly, although Gallop *et al.* did not insert any additional compounds between their final peptide and the encoding sequence, Janda *et al.* evaluated the addition of various linker types such as alkylated or pegylated units (Figure 2 B.). This careful screening showcased the superiority of pegylated units (in the form of a hexaethylene glycol) for optimal construction of the libraries. Remarkably, peg-containing linkers were conserved and remain the most abundant motif used in DEL synthesis almost 30 years later.

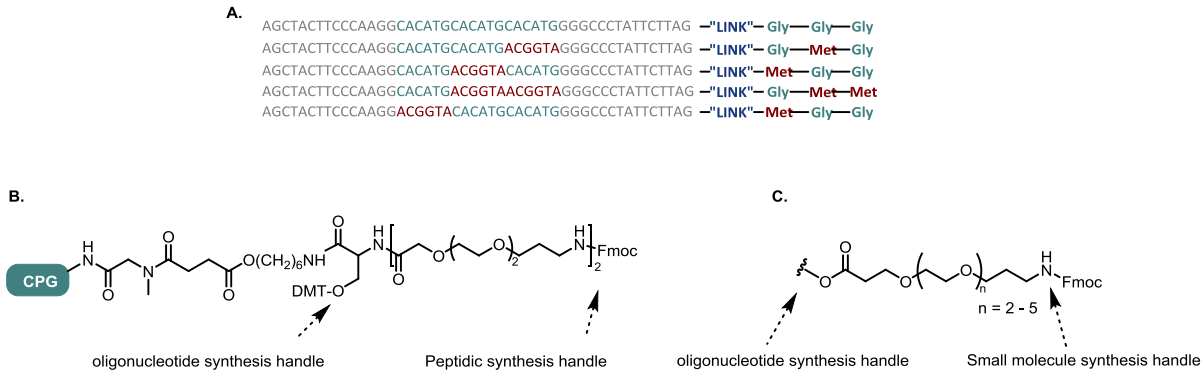

Figure 2. DEL linker designs. A. Original encoded library designed by Brenner and Lerner. B. Janda's first pegylated linker. C. AOP-NH_2 linker routinely used in DEL synthesis.

Nowadays, the most widely used DEL linker is the AOP-NH_2 (15-amino-4,7,10,13-tetraoxapentadecanoic acid), directly extended from the DNA headpiece (first section of the DNA tag) (Figure 2 C.). This linker is composed of several ethylene glycol units (from 2 to 5) and a short alkyl chain (from 2 to 5 carbon-long). The immediate interest of such linkers lies in the synthetic amenability. This completely inert chain provides a sufficient distance between the alternative small molecule and oligonucleotide building process and allows these key ligations steps to occur without mutual disruption. Nonetheless, the interest of such linkers also lies in the final application of DNA-encoded compounds, which is to be binding to a protein. Indeed, although the oligonucleotides used to encode small molecules in DELs are rarely exceeding 100 base pairs, they necessarily bring a consequent steric constraint in their vicinity, which can be alleviated by the presence of these long linear linkers. Benefitting from a comfortable space and being virtually "DNA-free", the potential small molecule can bind to protein sites without any appreciable disruption. However, no matter how inert these linkers can be, their inherent presence does have an impact on binding of compound as well as their physico-chemical properties. The nature of the bond between the linker and the small molecule obviously impacts the selection outputs, as well as the applicability of the library itself. In the next sections of this report, we will describe the different linkage strategies which has been developed, as well as their main applications.

II. Non-cleavable linkers

The term "non-cleavable linkers" designates all linkers connecting the small molecule to its encoding DNA tag during the whole DEL selection and downstream enrichment analysis process. These linkers must show obvious compliance to the reactions conditions to assemble the small molecules but also to the DNA tags successive ligations and final PCR to amplify the binders' sequences. It is therefore natural that long and inert pegylated chains were evaluated, [2] and later confirmed as being most relevant. [19]

Nonetheless, the nature of the terminal function on the linker, *i.e.* the reacting group to bind covalently the first building block to the encoding tag, determines the active state of the linker once the compound is built. Amino-modified pegylated linkers were first used for peptidic DEL synthesis which led to the formation of amide bonds between the linkers and the encoded small molecules. [19] The prevalence of peptidic library designs and the commercial availability of carboxylic acid bearing moieties (or corresponding activated or non-activated esters) naturally made of this amide bond the

most routinely used, [20] and many groups have turned their attention on the optimisation of this amide-bond formation for most DEL synthesis (Figure 3 A.). [21-23]

Although the predominant use of amino-modified linkers naturally led to the development of the amide bond formation, it's worth noting that the only few synthetic methods were developed for the formation of the amide bond in a reverse fashion, where the carboxylic acid moiety is initially located on the DNA strand (Figure 3 B.). Curiously, despite its interest, this method has proved to be particularly challenging, mainly because of poor stability of substrates and intermediates, as recently showcased by Neri and co-workers. [24] In their study, the authors could only react efficiently (*ie* conversions >70%) a third of a panel of diverse amines, under the optimized conditions. Naturally, several groups including ours, turned their attention on alternative pathways to tackle this reactivity hurdle. Warring's group approached the matter using a micellar approach, [25] which was previously employed to promote Suzuki couplings. [26] A careful screening of various headpieces proved that non-pegylated linkers were preferable to achieve optimal conversions. Despite the obvious added lipophilicity introduced by this linker, the strategy was proved to be particularly effective on a wide range of substrates in single and iterative cycles of synthesis, as well as being compatible with downstream PCR and sequencing. Although the alkyl chain could influence binding of the libraries to proteins, this study does bring a straightforward and scalable answer to the reverse-amide bond formation. Our group more recently took interest in the formation of such motifs, but throughout an indirect pathway. Indeed, we took advantage of the reactivity of terminal alkynes under copper-promoted Kinugasa reaction conditions. [27] In the presence of Cu(I) and a sacrificial nitrone, the formed on-DNA ketene intermediate can react with a variety of nucleophile in solution, notable primary, and secondary amines, to afford the corresponding amide. This strategy was proved to be particularly efficient on a wide range of amines, without the needs for any linker's optimization.

Although amide-forming reactions, in the direct or reverse fashion, do represent most linkers' binding strategies in the field, they do present unfavourable features regarding the binding of the formed on-DNA products to the targeted proteins. Indeed, DELs synthesis with conventional amide linkage usually starts with the first cycle of reaction, frequently with an amino acid, followed by a second and third cycle chemical conversion. With this approach, the chemical diversity created by the first cycle building blocks is enfolded by continuing Cycle 2, Cycle 3, and results in weakened interactions between these first cycle elements and the target proteins during DEL selection. In a study published in 2016, Arico-Muendel clearly showcased this phenomenon by demonstrating the low influence of Cycle 1 building blocks in many DEL campaigns' outcomes. [28] This phenomenon is likely the result of the inherent proximity of cycle 1 building blocks to the pegylated linker and their separation from the interface of protein binding sites. Nonetheless, this prompted us to develop alternative linker's design and reconsider the amide bond strategy. [29] We therefore opted to develop a pegylated linker equipped with a terminal tosylate function which can be directly engaged in alkylation reaction with a wide variety of nucleophile and notably primary amines, which can serve as an additional handle for the next reaction cycle (Figure 3 C.). Consequently, a branched motif can be formed without the need for any multi-functional cores and all building blocks cycle get a homogeneous influence on the binding to targeted proteins. Prior to this approach, several groups took interest in the alkylation motif in linkers designs. [23, 30] Naturally, the direct alkylation of terminal amino moiety can't be, in the presence of DNA or any type of oligonucleotides, conducted with even mild alkylating reagents. Nonetheless, a reductive alkylation strategy is perfectly compatible, which was exemplified both by Satz and co-workers in 2015, [21] using aldehydes and sodium borohydride as reactants, and by Baran's and Dawson's groups, which made use ketones or aldehydes and sodium cyanoborohydride under a RASS approach (reversible adsorption to solid support). [31] To complete the alkylation approach, direct arylation of amino-modified headpieces have been particularly employed mainly with trichloro-pyrimidine or triazine cores, [21] allowing for the easy formation of branched compounds (Figure 3 D.).

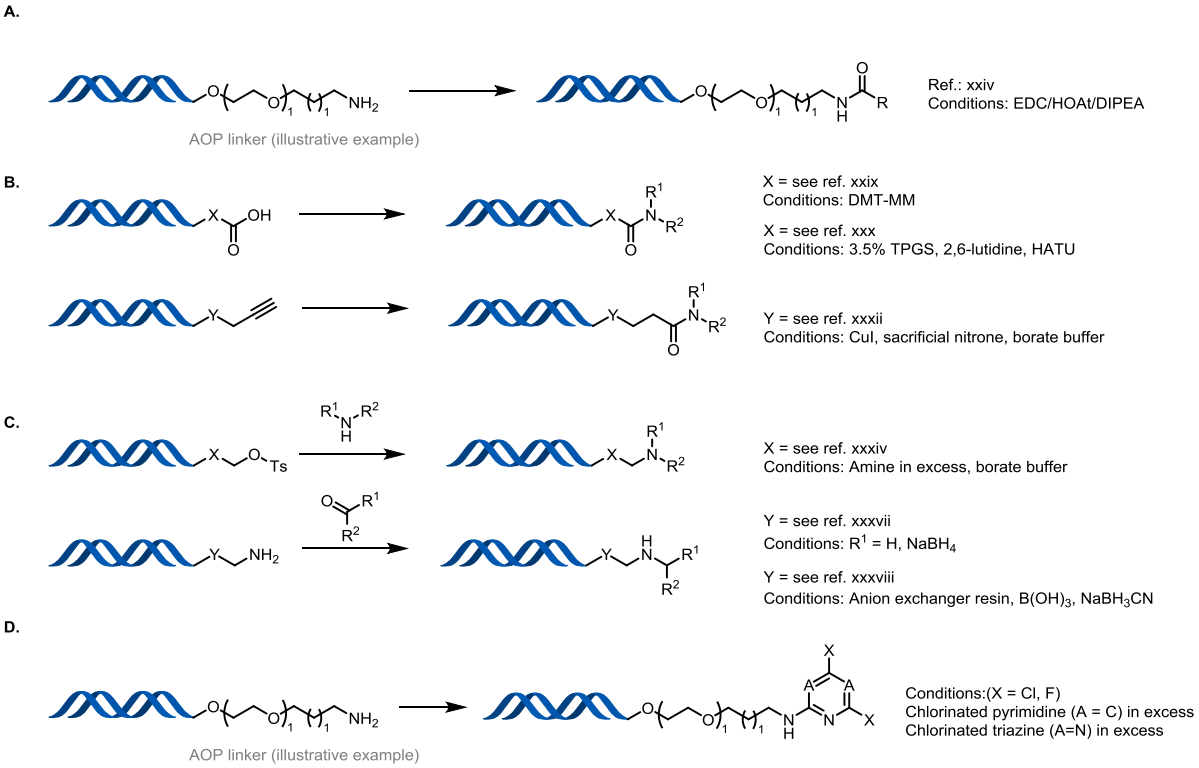

Figure 3. Non-cleavable DEL linker's motifs. A. Amide linker. B. Reverse amide linker. C. Alkylamine linker. D. Aryl linker motif.

A few other linker's motifs have been developed over the past years, most notably with sulfur-bearing moieties such as sulfonamides and sulfamides. Sulfonamides can be found in a variety of approved compounds and do represent a particularly relevant isostere of the amide bond, and despite initial attempts to conduct this transformation with sulfonyl chlorides (Figure 4 A.), [21] the relative instability of such reagents in aqueous media led Liu and co-workers to assess the efficacy of sulfinates, [32] which proved to be particularly competent. More recently, Baran's and Dawson's groups did apply a comparable approach using, once again, the RASS strategy (Figure 4 B.). [33] Parallelly, Sharpless *et al.* reported the on-DNA synthesis of sulfamides using iminosulfur oxydifluoride on amino-modified single stranded DNA headpieces. [34] Albeit no extension to double-stranded DNA or clear implementation in DEL synthesis was conducted, this initial report clearly proved the possibility to introduce such a biologically relevant motif on DEL linkers.

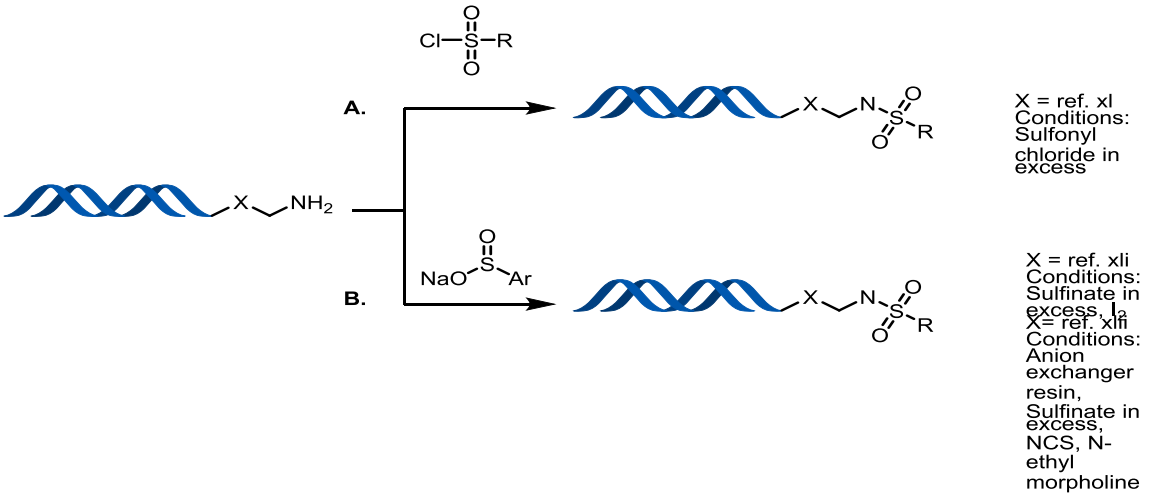

Figure 4. Sulfonamide motifs. A. Using sulfonyl chlorides. B. Using Sulfinates.

III. Cleavable linkers

On the contrary to non-cleavable linkers, constantly binding the on-DNA formed molecule and its encoding tag, cleavable linkers are only transient and can be cleaved upon well-controlled conditions to ensure both conservation of the integrity of both the small molecule and DNA. Over the years, their use has been limited to well-defined applications. Indeed, the founding principles of DEL requires a safeguard of the chemical information (the small molecules) embedded in the genetic information (the DNA tag), and it's no surprise that the simplest way to guarantee it is through covalent and long-lasting binding between the two. Nonetheless, the apparition of cleavable linkers in the early 2000's gave rise to fantastic technological advances in the field of DNA encoded library. In the following section, we'll give a brief overview of the major innovations and associated applications.

The first apparition of cleavable linker in DEL technologies can be attributed to Liu's group work on DNA-templated synthesis. [35,36] In this seminal study, the authors developed a particularly elegant method for building encoded peptidic library. In comparison to the typical split and pool approach, where the small molecule and its DNA tag are elongated almost independently, Liu's strategy is based on the ability of building blocks to react more efficiently and specifically with each other when they're both in proximity, itself ensured by the fact that both building blocks are conjugated to partially complementary DNA tags. Using a first building block conjugated to a DNA tag carrying all subsequent building block genetic encoding, the conjugation to a second building block is reacted to the first upon hybridization to the lead strand. Once the reaction between both building blocks is achieved, the corresponding complementary strands are annealed and the DNA tag encoding for the second building block is not necessary and can be cleaved, to follow up on the next reaction cycle (Figure 5 A.). To achieve efficient hybridization, ligation and final cleavage, the authors made use of a base-cleavable linker, (Bis [2-(Succinimidooxycarbonyloxy)ethyl] Sulfone), or BSOCOES, which can be bound to both and amino-modified DNA tag and any amino-bearing reagent. Upon basic treatment, both carbamate residues are decarboxylated and reform the initial amino-bearing starting materials (Figure 5 B.). This whole strategy, albeit suffering the comparison with the classic split and pool strategy in terms of numbers (higher numbers of compounds can be afforded with combinatorial synthesis), has the main advantage of being highly directed, providing libraries in considerably higher yields and purities. [37-40] The application of such principles to *in vitro* selection was rapidly envisaged and put in practice by the same group. [41-43]

Parallelly, a resembling strategy was developed by the Danish company Vipergen and rapidly implemented in DEL selection. Indeed, Gothelf and co-workers developed a cleavable linker with a reactivity close to BSOCOES, using succinimidyl 2-(vinylsulfonyl)-ethyl carbonate (SVEC) as the starting reagent (Figure 5 C.). [44] Easily installed on thiol-modified oligonucleotides, the linker can be conjugated to amino acids and efficiently cleaved under basic conditions. The implementation of this linkage into DEL technologies followed up quite rapidly as the same group reported the development of an innovative approach to DEL in 2009, [45] with the YoctoReactor®. [46, 47] Based on the DNA-templated synthesis principles, this approach lies on the formation of three or four-way DNA junctions with oligonucleotides linked with reacting building blocks, placed at the centre of these junctions and benefiting from the high local concentration to react efficiently. Once reaction is completed, the corresponding DNA strands (in obvious proximity as they're now linked by their corresponding building blocks) are ligated, and the SVEC linker can be cleaved off the second building block, leaving the formed compound on the lead strand to follow up with the next step of synthesis, until the desired product with the corresponding encoding is formed. This strategy does stand out from the split-and-pool synthesis by the extremely high fidelity and purity of the final libraries. Indeed, the double ligation approach (between building blocks and then corresponding DNA tags) allows to alleviate the risk of presence any incomplete intermediates which can easily be removed during intermediate purifications, thereby allowing to employ low yielding reactions, usually avoided in split-and-pool DEL synthesis. Based on these founding principles, new cleavable linkers were later developed for other applications. One noticeable example is the use of tetrahydropyranyl-based acid cleavable linker, developed by GSK and reported in 2021, for DEL-hits confirmation (Figure 5 D.). [48]

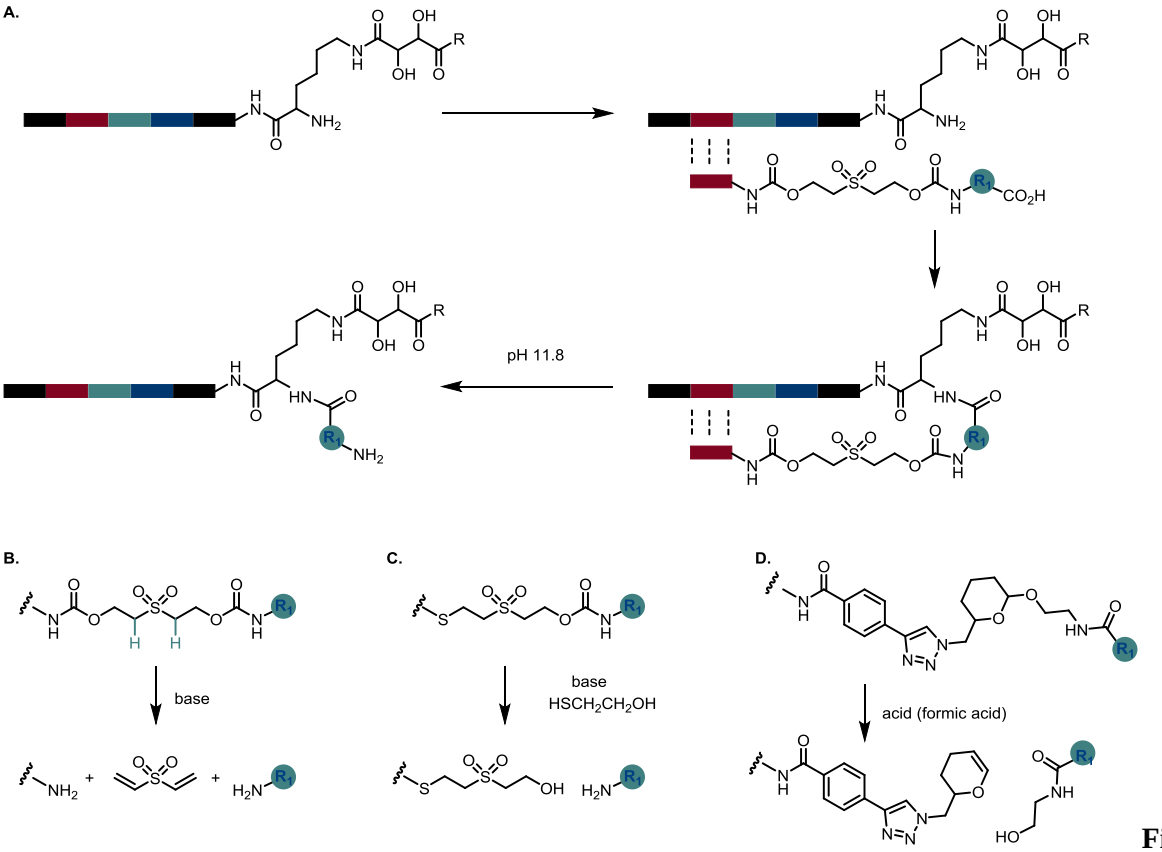

Figure 5. Cleavable linkers. A. DNA-templated synthesis using base-cleavable linker. B. BSOCOES-based linker. C. SVEC-based linker. D. THP-based linker.

In more recent years, several groups took interest in the diverse applications cleavable linkers can bring in DNA template synthesis and DEL technologies, notably with the development of photocleavable linkers. Indeed, if properly designed, cleavage of such compounds using UV does present the advantage of complete chemical orthogonality and milder conditions, therefore limiting the risk of unwanted products potentially obtained by cleavage reagents (Figure G). The first noticeable example of photocleavable linker in DEL technologies can be attributed to Li and co-workers in 2013, [49] who, taking advantage of the reactivity and compatibility of *o*-nitrobenzyl-based linkers described by Ordoukhanian *et al*., [50] demonstrated a particularly elegant templated strategy applicable to DEL synthesis. A similar approach was later used by Okamoto's group, [51] but most importantly by Paegel and co-workers, who greatly empowered the concept by developing activity-based DEL selection. Using this time, a one-bead-one compound (OBOC) DEL strategy coupled with a microfluidic set-up, the authors were able to encapsulate supported DNA-encoded compounds with protein targets. [52] Upon irradiation, the compounds can be released from their DNA-encoding tag and support and off-DNA interaction with the encapsulated target can be identified with induced fluorescence. "Active beads" can be isolated and corresponding DNA tags sequenced for hit identification. This approach was successfully used on autotaxin and later the receptor tyrosine kinase discoidin domain receptor 1 (DDR1), [53] before slightly derivatized for the discovery of novel antibacterial compounds. [54] Parallelly, a resembling photocleavable motif were also employed by GSK for DEL-hits confirmation, [48] to allow for direct off-DNA analysis using affinity selection mass spectrometry (AS-MS) of on-DNA hits obtain via DEL selection.

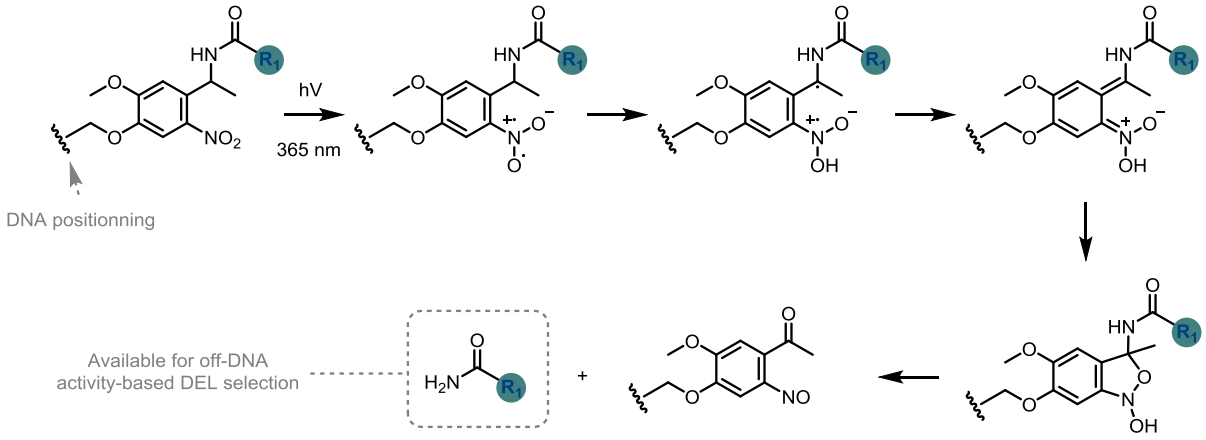

Figure 6. Photocleavable linker UV-triggered cleavage mechanism.

IV. Final remarks and outlooks

Throughout this review, we showcased the wealth of innovations on DEL linkers deployed by the scientific community since the inception of the technology. Albeit being relatively overshadowed by the spectacular advances of DEL-compatible chemistry for on-DNA synthesis of small molecule and DEL biology, the field did show drastic evolution since the early 1990's. A modest but encouraging array of ligation methodologies have emerged to vary the type of chemical linkage between the small molecules and their encoding tags, while cleavable linkers have showed amazing promises in modern DEL applications, showcasing ever improving versatility and orthogonality. As for DEL chemistry overall, these progresses have motivated new library designs and most importantly more advanced screening strategies using DEL principles. Considering the fast evolution of selection methods, [55-57] notably through the democratization of cell-based assays, the "niche" field of DEL linkers will undoubtedly still be thriving for years.

Challenges remain while old habits die hard. The prevalence of amide-bond formation, while still being a challenging reaction despite years of optimizations, perfectly illustrates the phenomenon. Notwithstanding it's obvious advantage, such as the commercial availability of gigantic collections of amine- and carboxylic acid-bearing compounds, this transformation truly conditioned the developments and adaptability of modern on-DNA chemistry for DEL synthesis. However, we have demonstrated in this report that many valuable alternatives have been developed and implemented. Alkylation, arylation, or sulfonylation to name only a few, could allow to introduce chemical diversity early in the DEL synthesis process and obviously influence DEL selection outcomes. Interestingly, many other motifs remain rarely explored and are worth mentioning.

Indeed, considering the expand of recent DEL chemistry advances, [58] a wide variety of reactions could be implemented on DEL linkers. Our group has notably been recently investigating the on-DNA reactivity of terminal alkynes which proved to be compatible with various homologation and heterocycle-forming reactions and could, [59-61] considering the already established workability of alkynes for azide-alkyne Huisgen cycloaddition, [62] and other heterocyclic-forming-reactions, [63] represent a particularly resourceful motif for DEL linkers (Figure 7 A.). Moving further, the use of carbohydrate motifs could also represent a particularly attractive strategy to drastically diversify DEL linkers motifs. In 2017, Flitch and co-workers have published a particularly insightful report on the workability of biocatalytic methods for on-DNA synthesis of carbohydrate libraries, [64] and despite the limited scope, this approach could be considered as a valuable starting point for further developments. Yet, lower-hanging fruits could be gathered immediately. Reactions such as guanidinylation, [65] or thiourea formation, [66] as well a more diverse arylations, either from amino-modified linkers with Buchwald-Hartwig or Ullmannn-type couplings could be considered for new linkers' designs. [31, 67-69] As well, the reactivity of aldehydes could be leveraged (Figure 7 B.). [58] Parallelly, although cleavable linkers have very recently showcased impressive improvements,

more simple motifs, such as disulfides, have not been thoroughly investigated. Our group recently reported an expedient protocol for disulfide formation on-DNA, [70] and considering the cleavage targetability of this bond, it wouldn't be surprising to witness its increased presence in future years.

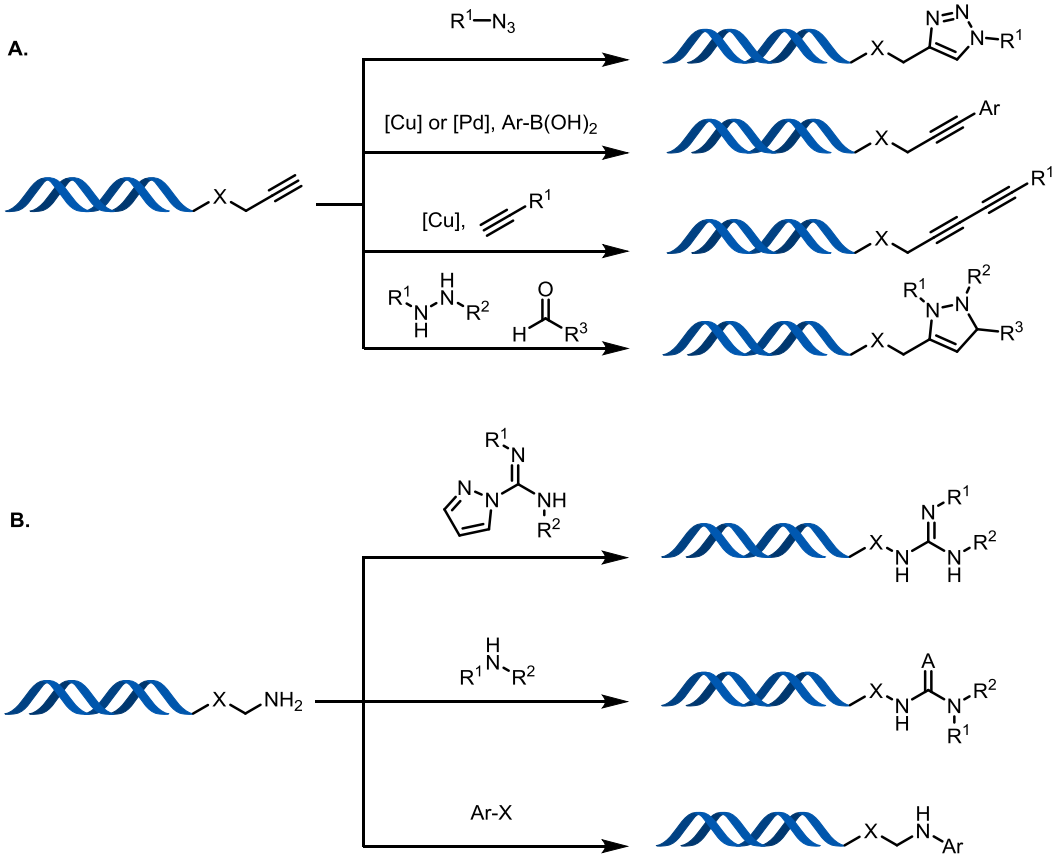

Figure 7. Potential new linkers motifs. A. Based on alkyne-modified oligonucleotides. B. Based on amino-modified oligonucleotide

For the past three decades, the field of DEL chemistry has been thriving, and we've witnessed remarkable advances which made of DEL a remarkable and most importantly amenable technology for hit generation in drug discovery. While progress on DEL linkers have been discreet, we've massively gained on flexibility, compatibility, diversity, and there's no doubt that they will gain increasing importance in the coming years. Several segments of drug discovery, such as molecular glues or target protein degraders, could truly benefit from the most recent developments in the field, and recent studies, such as Dou's recent work on the development of PROTAC®-dedicated DEL designs (including linker's), [71] is a good sample of the field of possibilities.

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