electrons line-up in one molecular orbital). This method is straightforward and simply involves the inclusion of more and more possible configurational line-ups into their wave function, permuting the electrons also into higher 'virtual' orbitals. Shaik and co-workers included as many as 200 million of those configurations in their full-configuration-interaction calculation. This leads to a wave function flexible enough to describe the multi-configurational character of the C_2 mojety. Their second strategy, applying valence-bond theory, does not rely on molecular orbitals at all and therefore is free from any assignments of 'electrons into orbitals'. Actually, the first quantum chemical calculation of a chemical system by Heitler and London in 1927 was based on this theory. In a typical valence-bond calculation, the input consists of strictly localized atomic orbital combinations (covalent and ionic valence-bond structures) familiar to chemists as Lewis structures. If one constructs these possible valence-bond structures adequately for all eight valence electrons in C_2, dynamic and static correlation is accounted for.

Having solved the electronic-structure problem Shaik and co-workers were finally ready to tackle the maybe even more challenging task: the determination of the bond order. Many disputes in the chemical literature rest on the dichotomy between the mere numerical solutions of quantum chemical calculations and their interpretation in terms of descriptive chemical concepts. Even right at the beginning of the quantum era, Erwin Schrödinger fiercely opposed the probabilistic interpretation of his strictly deterministic equation (actually, Schrödinger created his famous cat in order to ridicule the probabilistic Copenhagen interpretation)[6]. The concept of bond orders suffers from the same antagonism. Additionally, their definition and therefore calculation is by no means unique.

Historically, the concept of multiple bonds — or *verbundene Affinitäten* (bonded affinities) in the language of E. Erlenmeyer (1862) — between carbon atoms was rooted long before the development of quantum mechanics, it was even before the discovery of the electron. The concept was nevertheless retained into the post-quantum age, but the issue became even more delicate: In valence-bond theory, a bond order is given by the number of electron pairs in a weighted Lewis valence-bond structure. Applying the molecular-orbital approximation, different schemes of localization give rise to different — and there are many around — definitions of bond orders. Which definition should be applied? Using the canonical molecular-orbital scheme, the suggested bond order in C_2 would be 2, for example.

Recognizing this obstacle, Shaik and co-workers had to make a detour: the strength of the potential fourth bond was determined indirectly as the energy difference between two states: the full-bond state and the quasi-classical state, where the two odd electrons maintain only classical interactions (Fig. 1a). Doing so, they were after all able to bracket the strength of the fourth bond between 12 and 15 kcal mol^{-1}, which is much stronger than typical hydrogen bonds. Shaik and co-workers describe the fourth bond given as an "inverted bond", that is, additional to the well known σ and π bonds in acetylene, the outwardly pointing *sp* hybrids in C_2 are thought to contribute to their interaction too, through the two electrons pointing in opposite directions.

Still, there are some peculiarities in need of further explanation. The description of C_2 as a quadruply bonded molecule implies a pronounced restoring force that keeps the atoms in its equilibrium position (Fig. 1b). According to Shaik and co-workers, the mechanical bond strength, calculated as a compliance constant (relaxed force constant)[7], in C_2 is around 12 N cm^{-1}, just between the value of a typical C–C double (9 N cm^{-1}) and a C–C triple bond (17 N cm^{-1}). The authors explain this discrepancy in terms of an avoided surface crossing, however, this hardly accounts for the flamboyant slackness compared with a typical triple bond (30% decrease). The same holds true for the other diatomics between first-row elements studied by Shaik and co-workers, CN^+ BN and CB^-. Is the ground-state energy surface actually flattened near the minimum for these species? And what about the other candidates for a quadruple bond, for example N_2^{2+}? C_2 still seems not to have jumped from the test bench. ❐

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NUCLEIC ACID HYBRIDIZATION

Robust sequence discrimination

Careful consideration of thermodynamics has allowed the design of nucleic acid probes that are highly specific and virtually unaffected by changes in reaction conditions.

Grégoire Altan-Bonnet and Fred Russell Kramer

Hybrids formed by the binding of single-stranded oligonucleotides to complementary sequences are the strongest and most specific macromolecular complexes that can be formed. However, the ability to use hybridization probes to distinguish target sequences that differ from each other by as little as a single base (called a single-nucleotide polymorphism) has challenged assay designers for decades. Discrimination can only be achieved under carefully optimized conditions that control the salt concentration, oligonucleotide concentration, pH and temperature. If, for example the temperature is too high, very few perfectly complementary hybrids are formed, resulting in too little signal for reliable detection. Conversely, if the temperature is too low, mismatched hybrids also form, preventing discrimination from similar sequences. Dauntingly, small, natural variations create noisy environments that compromise reliability.

This narrow range of reliable conditions is a consequence of thermodynamic constraints. When two single-stranded oligonucleotides hybridize, energy is released by the formation of every base pair. Moreover, the complementary sequences are confined within a rigid double helix

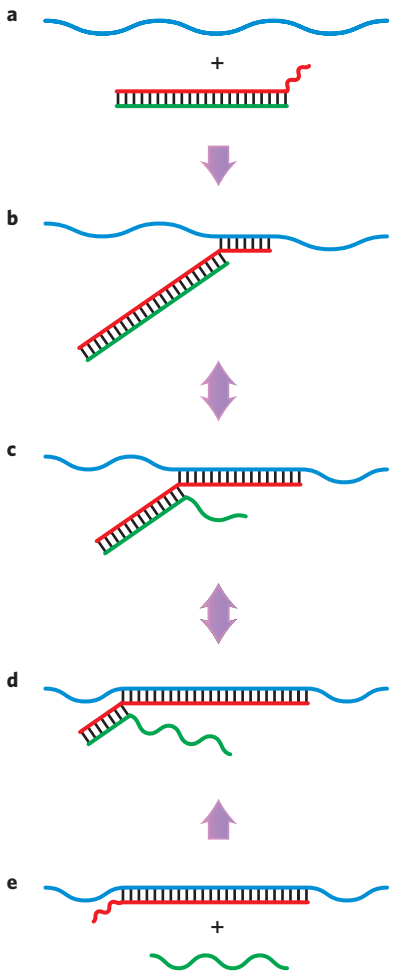

Figure 1 | Toehold exchange hybridization. **a**, Nucleic acid target strands (shown in blue) are incubated with oligonucleotide probes (red) that are bound to shorter probe complements (green), forming double helices that possess an overhanging probe segment. **b**, The overhang nucleates hybridization of the probe to the target, forming a 'toehold'. **c**, The length of the probe–target hybrid then increases and decreases by branch migration, resulting in a concomitant decrease or increase in the length of the probe–complement hybrid. **d**, Eventually, the probe complement is bound only to a short probe segment (the 'reverse toehold') that is not complementary to the target. **e**, The complement then disengages from the probe–target hybrid. As the number of molecules that are single-stranded and the number of molecules that are double-stranded remains the same, and the formation of additional base pairs is minimal, the thermodynamic free energy hardly changes, enabling the formation of probe–target hybrids under widely varying reaction conditions. Because the reaction is in virtual thermodynamic balance, the addition of only a few new base pairs drives the reaction forward. As a consequence of this design, toehold exchange reactions are exquisitely discriminatory against mismatched target sequences.

and cannot assume a variety of different conformations. Hybridization at low temperature is therefore accompanied by a large release of free energy that is similar in absolute terms irrespective of whether or not there is perfect complementarity; and at high temperature it is difficult to maintain the ability to discriminate targets that differ by a single base, because small changes in the reaction conditions alter the free energy released by every base pair that is newly formed, and alter the free energy released as a result of the conversion of randomly coiled single strands into rigid double helices.

For discrimination to occur over a much wider range of reaction conditions, assays are needed in which changes in conditions have a negligible effect on the free energy released by hybridization — the thermodynamic parameters must, by design, not be significantly altered when the probe strand binds to its complementary target strand. Such a design has been explored in detail by David Zhang and his colleagues at Harvard University, and the exhilarating experimental results are now reported in *Nature Chemistry*[1].

In their assay (Fig. 1), the oligonucleotide probes are, for example, 29 nucleotides in length, and are initially hybridized to shorter 'probe complement' oligonucleotides that are, for example, 22 nucleotides in length — forming a 22-base-pair double helix with a seven-nucleotide overhang. When single-stranded targets are added to the reaction mixture containing these constructs, the overhanging sequence first hybridizes to the target sequence, nucleating the process of forming a longer probe–target hybrid. After this toehold is established, the probe–target hybrid can extend in length. However, for each additional base pair formed between the probe and the target, a base pair between the probe and the probe complement must dissociate. The probes themselves are thus referred to as toehold exchange probes. Probe–target hybrid formation occurs in a reversible manner. That is, the hybrid can increase or decrease in length and this three-molecule (probe–complement–target) hybridization complex continuously alters its configuration, undergoing a 'random walk', until the process is completed by either the probe falling off the target and returning to the embrace of the complement, or by the complement falling off the probe, leaving the probe hybridized to the target.

Here is the beauty of this probe design: only the 24 nucleotides at the end of the 29-nucleotide probe are complementary to the target. The additional five-nucleotide sequence on the other end of the probe is complementary only to the probe complement. So when the probe is bound to its target and free of the complement, this five-nucleotide segment remains single stranded and can serve as a 'reverse toehold'. By design, however, the probe–target hybrid is two-base pairs longer than the probe–complement hybrid, so the probe–target hybrid is slightly more stable and the reaction favours its formation.

In this way, hybridization between probe and target has virtually no net thermodynamic effect: the number of double-stranded and single-stranded molecules does not change, and importantly, each time a probe binds to a target forming 24 new base pairs, 22 other base pairs are eliminated. The hybridization reaction is driven by the two-base-pair increase alone. Unlike classic assays, very little net change occurs in a toehold exchange reaction, so alterations in reaction conditions have an insignificant effect on the ability of the probes to form probe–target hybrids.

Thermodynamically, toehold exchange reactions are designed so that the release of free energy on probe–target hybridization is minute, thereby significantly increasing sensitivity to the presence of mismatches[2]. The presence of a single-nucleotide polymorphism in the target sequence reduces the driving force for hybrid formation, because only one additional base pair is formed — providing insufficient energy to overcome the destabilizing effect of the mismatch. Consequently, toehold exchange reactions robustly discriminate between perfectly complementary targets and targets containing a single-nucleotide polymorphism. In a classical assay a single-nucleotide polymorphism is detected as the result of forming, say, 23 instead of 24 new base pairs — a 4% difference; whereas in a toehold exchange reaction, the same polymorphism is detected by forming one instead of two new base pairs — a difference of 100%.

Toehold exchange probes are the purest example of probes that have alternative stable states. Robust discrimination is dependant on the near-thermodynamic equivalence of these states[3]. Earlier examples of probe designs that incorporate this idea include stringency clamps[4], molecular beacons[5] and strand-displacement probes[6]. These earlier probe designs, however, require careful 'tuning' and optimization to achieve the desired results, whereas toehold exchange probes are optimal by design.

There is, however, a price to pay for the higher specificity of toehold exchange reactions (and other reactions employing conformationally constrained probes): the reaction kinetics are slower. The use of decoy structures that compete with the desired probe–target hybrid at the thermodynamic level introduces a large activation-energy barrier. For target strands that are present

in micromolar concentrations, equilibration with specificity takes up to three hours. By comparison, unconstrained probes associate with their targets in seconds, albeit with lower specificity. With toehold exchange reactions, speed is sacrificed to maximize specificity.

Toehold exchange reactions will be employed in many practical applications. One such use is for multiplex diagnostic assays, where the simultaneous quantitation of different target sequences provides useful clinical information. In these assays, the probe strands contain a fluorophore that emits a characteristic signal, and the probe complement strand carries a fluorescence quencher. When probe and complement are in close proximity, fluorescence is 'switched off'. When the probe binds to its target, however, the quencher is dissociated and a bright fluorescence signal results. The simultaneous use of different toehold exchange probes, each specific for a different target, and each labelled with a differently coloured fluorophore, will enable the design of robust multiplex assays[7].

Another application might be the detection of rare mutants among many copies of a wild-type target in a clinical sample (as is the case when a few cancer cells are present in a sample containing many normal cells). In this situation, the toehold exchange probe could serve as a primer for a gene-amplification reaction, so that the mutant is selectively amplified. Amplification assays employing toehold exchange primers could thus replace more complex and time-consuming sequencing procedures.

Most excitingly, nanoscale structures can be formed and altered through programmed interactions between nucleic acids that involve toehold exchange mechanisms[8]. These interactions can be catalytic[9], and can serve as the elements of logic gates, which can be combined into complex analytical circuits[10]. Within these functional assemblies, all the different hybridization reactions need to be highly specific and insensitive to changes in reaction conditions to work well together. Toehold exchange probes are ideal for these applications, because they are simple to design, selective in their interactions, and thermodynamically robust. ❐

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ULTRAFAST CHEMICAL PHYSICS

In search of molecular movies

Ultrafast chemical physics follows in the explosive wake of technological innovation, using light and radiation sources to study phenomena at timescales where the boundaries between physics and chemistry dissolve. UCP 2011, the second meeting in a series, explored the current state of the art in ultrafast time-resolved spectroscopy.

Julia A. Weinstein and Neil T. Hunt

The fascinating world of ultrafast chemical physics (UCP) has seen rapid development in the past two decades. The UCP field quickly embraces modern advances in 'ultrafast' methods and uses them to study the intimate mechanisms of chemical processes from the sub-atomic level upwards, in all states of matter, and across many timescales (Fig. 1). The techniques used allow real-time insights into a range of phenomena, from individual atomic motions and bond-making/breaking processes, to how large biomolecules and molecular motors function. The recent UCP conference (www.ultrachemphys.org), held at the University of Strathclyde in Glasgow, 14–16 December 2011, aimed to provide an overview of the current state of this field.

The conference themes for UCP 2011 were broadly based in order to facilitate the exploration of diverse areas. The meeting began with a series of overview talks that discussed the current developments in multidimensional spectroscopy (Klug, Imperial College), ultrafast X-ray diffraction (Först, University of Hamburg), terahertz spectroscopy (Wynne, University of Glasgow) and attosecond science (Tisch, Imperial College London). It was clear that the more established areas such as multidimensional and terahertz spectroscopies are beginning to move beyond the realms of technique development towards a critical evaluation of which fields the methods can be of most benefit to. On the other hand, techniques such as those featuring femtosecond X-ray diffraction and attosecond spectroscopy are still evolving as shorter, more intense laser pulses and synchrotron radiation become available.

Multidimensional spectroscopy[1–3] has progressed significantly following its inception around a decade ago. It uses a correlation of excitation and detection frequencies to spread an ordinary absorption spectrum — UV/visible or infrared — over a second axis, providing information on electronic and vibrational couplings and time-resolved dynamics. Close parallels can be drawn between these methods and the most successful multidimensional spectroscopy — nuclear magnetic resonance. A principal advantage of multidimensional optical/infrared methods over multidimensional NMR is ultrafast time resolution, which is mainly limited by the laser pulse duration. Multidimensional spectroscopy is a powerful method for resolving the role of vibrational coupling in energy-dissipation pathways, so as to understand the role of individual structural motifs, individual vibrations, and solvent in structural dynamics and reactivity. Recently, transient multidimensional infrared and UV/visible spectroscopies have begun to develop, permitting the study of the structural dynamics of non-equilibrium, electronically excited states and thus providing new insights into photoreactivity.

The meeting highlighted that multidimensional spectroscopy is extremely useful for gaining insight into a diverse range of topics including the structure of water or the action of photoacids (Nibbering, Berlin)[4] charge-transfer processes (Sazanovich and Weinstein, University of Sheffield)[5] and water