# High-Throughput Combinatorial Synthesis of Stimuli-Responsive Materials

*Alisa Rosenfeld and Pavel A. Levkin**

**Stimuli-responsive materials find wide applications in different biological and medical settings. Traditionally, stimuli-responsive materials are synthesized and evaluated individually one-by-one. The drawback of this approach is the scarceness of possible combinations that can be practically tested for the purpose of saving time, consumables, and manpower. High-throughput methods are therefore important to accelerate the discovery of stimuli-responsive materials and to screen for biological interactions of interest in parallel. The objective of this article is to provide an overview of the successful employment of combinatorial high-throughput synthesis and screening of stimuli-responsive materials. In particular, these include thermoresponsive and hydroresponsive materials. Advantages of a combinatorial approach as well as of utilizing high-throughput methodologies in the development of stimuli-responsive materials are reviewed. Possible evolution trends of stimuli-responsive materials, advanced by high-throughput methodologies, are discussed.**

## 1. Introduction

Stimuli-responsive materials, also called smart or adaptive materials, are attracting growing attention in different fields, including biotechnology and medicine, as these materials are able to respond to minute changes in their surrounding environment by changes in their chemical, physical, or biological properties. By applying a specific stimulus, stimuli-responsive materials can be altered at molecular scale in different ways (e.g., conformational transition, hydrophilicity, charge, solubility, chemical bond cleavage, etc.), resulting in changes in the materials' macroscopic features such as changes in volume, shape, performance, wettability, color, mechanical motion, release/capture of molecules, degradation, aggregation, precipitation, etc. A plethora of stimuli can be used to trigger a specific material response, and corresponding materials are comprehensively reviewed in the literature. These stimuli include temperature,[1] light,[2] mechanical forces,[3] electric and magnetic field,[2b] ultrasound,[2b,3] pH,[4] ionic strength,[5] humidity,[6] various small molecules (e.g., glucose,[7] $CO_2$,[8] and redox agents[9]), enzymes,[10] and some more complex combinations of stimuli.[11]

Sophisticated diverse examples of stimuli responsiveness and adaptivity are often found in natural biological systems. In the course of evolution and in order to interact with their dynamic environments, biological systems have acquired various complex responsive, adaptive, and dynamic properties. On every level of life, from dynamic ecosystems down to tissues, cells, and biopolymers, responsiveness plays a crucial role and is a remarkable and ubiquitous biological property (sperm chemotaxis toward the egg, gravitropism of plant roots, and coral reef response to sea temperature as an illustration). A bioinspired design of responsive synthetic materials can benefit from millions of years of evolution and is an important means of accelerating the development of functional materials, improving their performance, and for fundamental research. For example, self-healing is an omnipresent feature of living organisms that has inspired the development of self-healing smart materials.[12] Bioinspired routes for preparing stimuli-responsive materials can, for example, use enzyme-cleavable linkers to achieve degradation of a polymer network in the presence of a corresponding enzyme.[10] Self-oscillating materials based on the Belousov–Zhabotinsky reaction[13] carry an analogy to autonomous oscillation in living systems like heart contractions and pulsatile secretion of hormones. However, they stand apart from other responsive materials, as they oscillate in a self-governed manner and need no oscillating external stimuli.[14] Multifunctional materials can be also rationally constructed by incorporating building blocks with known responsive behavior (e.g., pH-responsive and temperature-responsive functional groups). They reveal predictable, combined characteristics (e.g., pH- and temperature-responsiveness) that are independently traceable to installed building blocks.[11a]

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## 2. Benefits of Combinatorial High-Throughput (HT) Methodologies

Many high-end technologies, such as tissue engineering,[15] soft robotics,[16] sensors,[17] etc., require responsive materials

perfectly adapted for each application. Nonetheless, the functionality and tempo of development of new responsive materials remain far behind the broad-ranging demands of the aforementioned fields. To fulfill the miscellaneous needs and realize novel applications, we need to overcome the undersupply of responsive materials by covering larger chemical space and creating new materials faster. Combinatorial methodology is a powerful approach to quickly generate a vast number of structurally and functionally diverse materials using just a few functional building blocks (**Figure 1**A). A multitude of design parameters and an even larger number of their combinations can essentially contribute to the precise fine-tuning of a responsive material for a specific application. However, stimuli-responsive materials are usually synthesized and evaluated iteratively one-by-one. The drawback of this approach is the paucity of possible combinations that can be practically tested for the purpose of saving time, consumables, and manpower. This forces researchers to follow the beaten track—to investigate only some of the commonest and most obvious combinations of triggers and responses, and to change only one parameter at a time, despite the large variety of possibilities. Therefore, although a considerable amount of research is being devoted to the field of responsive materials, the actual development of such materials remains slow and inefficient and the design principles, including structure–function relationships, are often not fully understood.

To overcome slow and inefficient, one-by-one synthesis and screening, miniaturization, parallelization, and HT methodologies must be employed (Figure 1B). HT synthesis and screening proved themselves initially as a powerful strategy in the pharmaceutical industry; HT syntheses and screenings have recently been applied in material discovery.[18] Besides saving time, consumables, and work force, there are several other considerable benefits of employing combinatorial HT synthesis and screening in responsive material development. For rationally constructed materials possessing predictable characteristics, the combinatorial HT approach can enhance their focused screening and optimization. By varying known design parameters, rapid material fine-tuning for a particular application is achievable, and varied parameters can be mapped, providing insights into structure–function relationships. Yet, with the growing number of components being introduced, it becomes difficult to predict or rationally design the interplay between them. Combinatorial screening over a multitude of building blocks can reveal inhibitive (retarding) or cumulative (synergetic) effects that are otherwise unpredictable. This is particularly useful for deepening our understanding of synergetic and antagonistic interactions between different building blocks. Combinatorial HT screening of random libraries is therefore particularly useful if the ability to rationally design a responsive material is limited by theoretical knowledge that does not happen to be available. For example, the sheer complexity of the extracellular matrix and rather seldom predictability of mutual cell-material interactions make combinatorial methods and HT strategies powerful tools in stimuli-responsive material synthesis for tissue engineering applications. An intrinsically HT method also encourages the employment of counterintuitive, creative combinations of starting materials to discover materials with novel properties, functions, and responses. In light of the unpredictability of mutual component interactions and large, random (diversity-based) sets of combinations, combinatorial HT synthesis, and screening offer a convenient opportunity to explore a variety of properties and functions that are accessible only with great effort in one-by-one synthesis or that cannot be rationally designed. In doing so we can transform a vast quantity of combinations and components into novel quality. Screening random libraries can therefore be performed to pursue novel discoveries, to acquire a key competitive advantage in what has become an aggressive environment of patenting policies.

In this progress report we aimed to review selected state-of-the-art examples of applying combinatorial HT synthesis and screening to accelerate the development of responsive and adaptive materials. We also included big libraries (>60 members) made in combinatorial but low-throughput one-by-one manners. The scope of this progress report will be limited to organic polymers and long oligomers, thus differentiating from widely reviewed HT synthesis of peptides, small molecules and the like, despite some interesting contributions were made to screenings of supramolecular photoresponsive hydrogels.[19]

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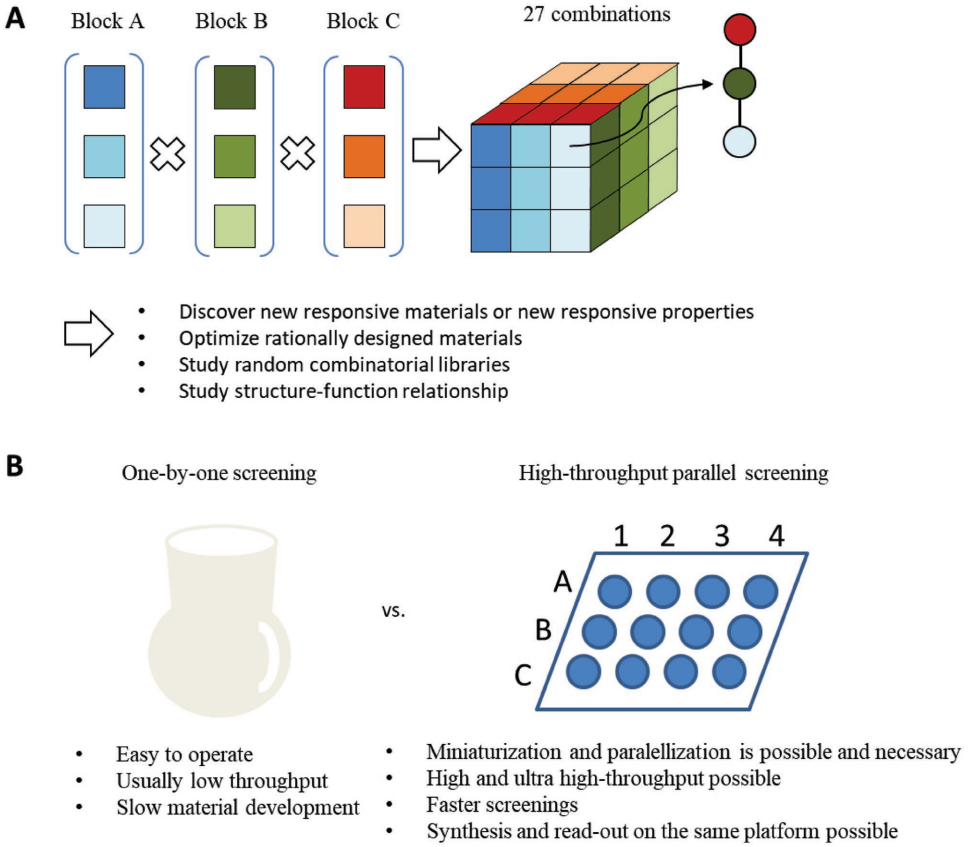


**Figure 1.** A) Combinatorial approach allows quick generation of chemically diverse responsive materials and their investigation. B) Stimuli-responsive materials are usually synthesized sequentially one after another; therefore, the number of testable combinations is limited. High-throughput techniques combined with the combinatorial approach enable us to cover otherwise inaccessible chemical space, and make possible material optimization for fundamental research and the discovery of novel properties.

## 3. Combinatorial High-Throughput Synthesis of Stimuli-Responsive Materials

Thermoresponsive materials are one of the most well-studied and understood stimuli-responsive materials, including the most thoroughly investigated poly(*N*-isopropylacrylamide) (pNIPAAm). Its responsive properties have been extensively exploited in various biological applications, e.g., for controlled, temperature-triggered cell binding and release.[20] There are several examples of combinatorial HT synthesis of thermoresponsive materials. Combinatorial HT synthesis of thermoresponsive materials for mild cell release was established by Bradley and co-workers in 2009 (**Figure 2**A,B).[21] Water soluble *N*-isopropylacrylamide (NIPAAm) and *N*,*N*-diethylacrylamide (DEAA), that are known to confer thermoresponsive properties to final hydrogels, as well as other monomers were used to produce a 2280-membered library on a single glass slide via inkjet printing. In the initial screen, the hydrogels were quantified in terms of their cell-binding and cell releasing properties upon cooling from 37 to 20 and 10 °C. Following the initial screen, 23 "hit"-hydrogels were reprinted in "hit"-microarray in multiple copies, and their cell-binding and cell-releasing properties were assessed regarding the average area of a hydrogel spot. Finally, three best hydrogels were scaled up and scrutinized one-by-one. Their study revealed that these hydrogels offered strong cell-detachment efficiency: up to 91% of cells could be released upon a thermal switch with the high viability of released cells (>85%). Being shrunk and dehydrated at 37 °C, the hydrogels swell at lower temperatures, resulting in cellular detachment. A subsequent screen of "hit"-microarray on five cell lines (HeLa, L929, HEK-293T, mouse embryonic stem cells E14tg2a, and B16F10) enabled the discovery of an optimal cell binding/releasing material for each cell type. The ability of NIPAAm-based thermoresponsive materials to release cells in response to temperature changes was exploited by Bradley and co-workers to identify a long-lasting cell-adhesive material that permitted the much-in-demand, gentle, reagent-free harvesting of human embryonic stem cells (hESCs). After their initial discoveries,[21] Bradley and co-workers synthesized an arrayed library of 609 hydrogels from 18 monomers.[22] In their initial screens, hESC attachment, viability, and the marker expression of cells attached to hydrogels were studied in an HT manner, then the best 25 polymers were scaled up and tested to see how efficient the thermally-triggered cell release was. Their study ultimately resulted in a chemically well-defined hydrogel that supported the long-term growth of hESCs while preserving their pluripotency and enabling nondestructive serial passaging and dissociation via thermal modulation and excellent (>90%)

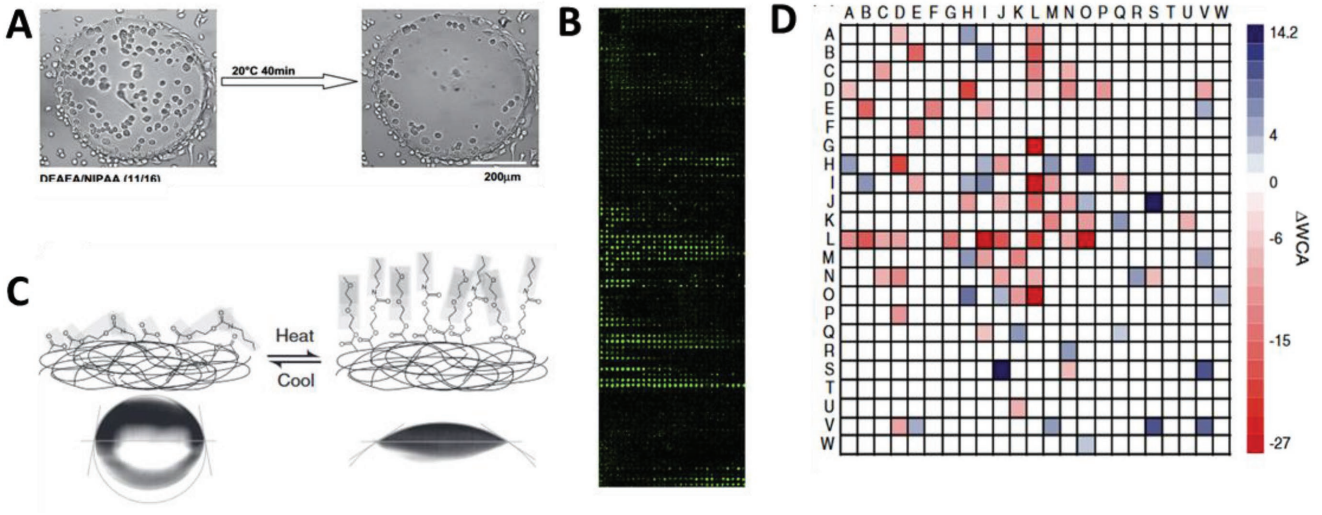


**Figure 2.** Examples of readouts for measuring thermoresponsiveness of materials compatible with high-throughput combinatorial screenings: cell adhesion or detachment and wettability changes upon cooling or heating. A) Bright-field image showing cell detachment from a polymer spot upon cooling. Reproduced with permission.[21] Copyright 2009, Elsevier. B) Fluorescence image of a 2280-membered library with captured fluorescent HeLa cells; cell numbers calculated at different temperatures correlate with polymer composition and temperature. Reproduced with permission.[22] Copyright 2013, Nature Publishing Group. C) Schematic depiction of conformational changes in an exemplary copolymer upon temperature changes, which causes changes in WCA. Reproduced with permission.[23] Copyright 2013, John Wiley & Sons. D) Intensity map of WCA changes upon heating of a polymer library, letters indicating the monomers mixed at 50/50% ratio. Reproduced with permission.[23] Copyright 2013, John Wiley & Sons.

releasing capacity. These discoveries reveal the hidden potential of the combinatorial HT synthesis of responsive biomaterials and HT assessment of their biological properties. The design of the libraries was based on incorporating NIPAAm to confer thermoresponsive properties. However, with the number of monomers they used, it was difficult to rationally predict, but easy to test the properties of each unique polymer. The resulting diverse, large libraries made it possible to rapidly identify and optimize biocompatible materials with specific responsive properties. Without miniaturization and parallelization, the screening of hundreds of materials would require not only large quantities of chemical precursors but also an enormous amount of expensive stem cells and is thus quite unrealistic.

So far, thermoresponsiveness has been measured by the number of cells released from the material after the temperature modulation. An essentially different cell-free approach to define thermally responsive materials was also established based on temperature-correlated changes in wettability (Figure 2C,D).[23] HT measurements of the water contact angle (WCA) were taken to identify materials with thermoresponsive properties among 279 unique materials arranged in array format; subsequently, time-of-flight secondary ion mass spectrometry (ToF-SIMS) enabled the detection of temperature-dependent molecular conformational changes in "hit" materials. The two polymers identified in this study later proved themselves as competent materials for thermally triggered *Escherichia coli* release, revealing noteworthy (up to 96%) efficiency, with possible application in self-cleaning bacterial filtration systems.[24]

Polymer microarray technology was recently employed to discover a responsive material able to alter an incorporated dye's fluorescence upon a shift in temperature.[25] An array of 275 polymer features in quadruplicates was produced by inkjet printing and differentiated in terms of their fluorescence enhancement or quenching of entrapped fluorescent dye. Five representative combinations were up-scaled to produce polymer beads to further investigate opto-thermoresponsive properties at temperatures ranging from 25 to 55 °C.

Another cell-free approach, turbidimetry, was used to scrutinize thermoresponsive behavior of polymers. Becer et al. synthesized a library of 60 oligo(ethyleneglycol)methacrylate-based polymers (partially in automated parallel and partially in individual one-by-one manner); 32 polymers were subjected to turbidimetry to investigate the responsive properties by measuring the cloudy point at different pH values, the pH-responsiveness being attributed to the incorporation of acidic monomer units.[26] Interestingly, several copolymers exhibited both thermo- and pH-responsiveness, although their homopolymers revealed no lower critical solution temperature behavior. This effect was only detectable by systematic random library screening of counterintuitive monomer combinations, thus showing the importance of such screenings to find unexpected, novel effects.

Water is a ubiquitous solvent and is conveniently applicable as a trigger of various responses. An atomic force microscopy (AFM) surface high-throughput characterization study of a 576-membered acrylate library revealed four different topographies (flat surfaces and surfaces containing pits or nodules or particles). Pits were only observed in copolymers of 4-*tert*-butylcyclohexylacrylate with either (oligo)ethylene glycol acrylate or 3-(dimethylamino)propyl acrylate. The difference in hydrophilicity of these monomers leads to a phase-separation and therefore to pits, composed of hydrophilic monomer, dispersed in bulk hydrophobic monomer. Almost all of pit-polymers exhibited a rapid pit-to-bumpy transition upon exposure to water (**Figure 3**).[27] These transitions, based on swelling of hydrophilic pits, were almost all reversible for at least one wet-dry-wet cycle. Remarkably, this

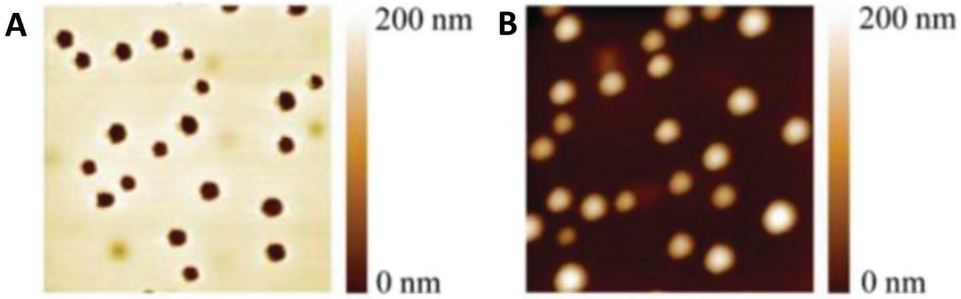


**Figure 3.** A 576-membered acrylate library, constructed from chemically very diverse monomers with different hydrophilicity, was characterized by HT-AFM and revealed four different topographies, including a nanostructured pit topography (A). Such polymers were discovered to be hydroresponsive and deliver a bumpy topography (B) upon immersion in water, based on swelling of phase-separated hydrophilic pits. Reproduced with permission.[27] Copyright 2011, Royal Society of Chemistry.

interesting response was only detectable via HT analysis methodologies, emphasizing their importance in high-throughput smart material development. The materials could probably not have been discovered if smaller sample sets had been used. This example accentuates the compatibility of combinatorial HT synthesis and screening methods to discover novel responsiveness and stresses the importance of random (diversity-based) combinatorial HT screening for discovering novel materials.

Hydrolytic degradation is an important parameter in various biological settings. Typically, ester linkages are installed to induce hydroresponsiveness under biological conditions. Langer and co-workers investigated hydrolytically labile poly-(*β*-aminoesters) as promising synthetic transfection vectors by first synthesizing a 140-membered library in vials,[28] followed by half-automated 2350-membered library synthesis in 96-well plates.[29] From the first screening two polymers (≈1% from the library) and from the second screening 46 polymers (≈2% from the library) exceeded the performance of conventionally employed synthetic transfection agent poly(ethylene imine). The significantly larger second library, enabled by automatization strategies, delivered more "hits," having almost the same success rate as the first library, emphasizing the importance of the throughput. Bradley and co-workers investigated another class of hydrolytically labile materials and synthesized a 61-membered library of poly(*ε*-caprolactone)-based polyurethanes in bulk, and then in form of polymer microarray to identify 57 copolymers that facilitate cellular attachment and growth.[30] Hydrolysis experiments were performed in bulk on a larger scale on certain representative materials and demonstrated faster degradation of more amorphous structures than semicrystalline copolymers. Notably, the hits from both studies were selected and optimized in terms of their water solubility and gene delivery ability or cell responses, respectively, and not in terms of their hydroresponsiveness or degradation properties. Combinatorial HT (random) screening can be particular useful for investigating these features, as the mechanism of the degradation and its determining parameters (e.g., polymer hydrophobicity, pH, and temperature of aqueous solution) are not always known, and the kinetics of degradation/rate of hydrolysis can be adapted and optimized for specific clinical applications (e.g., drug delivery or tissue engineering) by manipulating the material composition.[31] This issue was addressed by Burdick and co-workers.[32] A 120-membered library of photocrosslinked, hydrolytically labile poly-(*β*-aminoesters) was synthesized and characterized in terms of degradation properties, albeit in a one-by-one manner (**Figure 4**) and later screened against bulk properties, cell toxicity, and attachment to identify an osteoconductive material.[33] The screening over a multitude of starting material combinations has proved the importance of the combinatorial approach, as a hydrolytical degradation trend cannot be easily derived from the chemical structure of educts. Combinatorial HT synthesis and screening has great potential and should be used to investigate other structure–function relationships and triggers. An 80-membered 2-oxazoline-based library including 40 potentially degradable poly(2-oxazoline)-*co*-polyesters was synthesized in bulk. To lay a foundation for future experiments on correlation of hydrophobic character of the gels with the degradation rate, as a proof of concept authors have tested one exemplary polymer to prove the pH-dependency of degradability in water as well as the degradability by esterases.[34] The degradation rate was evaluated by quantification of released Eosin B, which was incorporated prior to polymerization, and was found to be the highest at pH 8 and in the presence of porcine liver esterase.

Interestingly, some efforts were made to combine hydrolytical lability with UV-triggered degradation, albeit in a one-by-one manner resulting in small libraries.[35] Combinations of triggers demand more intense investigation. Such research can be accelerated by combinatorial HT synthesis.

## 4. Summary and Outlook

The ability to change physical properties as a response to changes in the physical environment makes stimuli-responsive materials promising substrates for the controllable modulation and manipulation of diverse biological systems in not only tissue engineering, drug delivery, bioseparation, but also biosensors and bioactuators. Of special interest for biological applications are physical stimuli, such as light, temperature, electric and magnetic fields, because the material response can be triggered remotely in a noncontaminating way, often enabling spatial and temporal control.[2b] Combinatorial HT synthesis applied to the field of stimuli-responsive materials is particularly useful in the rapid identification and refinement of "hit" structures, in studying structure–function relationships, and in

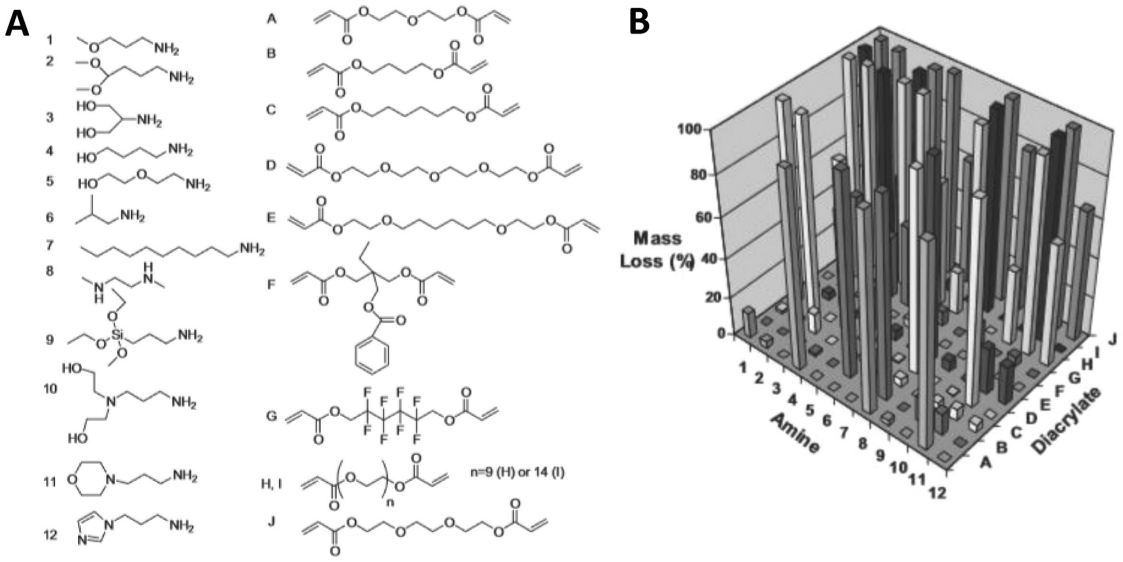


**Figure 4.** A) Acrylates and amines used to synthesize a library of polymerizable molecules, which then were used to form a library of hydrolytically labile polymers. B) Degradation behavior of the polymers based on the presence of ester linkages was detected by mass loss after one day of incubation in phosphate buffered saline at 37 °C; faster degradation was attributed to hydrophilic diacrylates, whereas slower degradation was attributed to hydrophobic amines. Reproduced with permission.[32] Copyright 2016, Wiley VCH.

mimicking biological systems irrespective of the eventual lack of theoretical knowledge, while seeking novel responsive functions. Surprisingly, despite the various potential applications of responsive materials and the ease of creating a combinatorial library, very few examples of combinatorial HT synthesis of stimuli-responsive materials have been published to date. The field is essentially still in its infancy, and it is often not considered a method of choice despite its obvious advantages. The following challenges related to the implementation of high-throughput combinatorial synthesis of smart materials still remain: 1) many laboratories miss appropriate automatization and miniaturization equipment, such as liquid dispensers; 2) using some conventional analytical methods, e.g., NMR, to analyze materials in a high-throughput manner is not trivial; 3) material microarrays, for example polymer microarrays, often lack the possibility to encapsulate cells or other microorganisms; 4) the added value of screening large random combinatorial libraries is underestimated, which results in intentionally small libraries. As all the studies we have cited were published in the last two decades, the vast potential of combinatorial HT synthesis is still waiting to be discovered. A deliberate employment of combinatorial HT synthesis instead of classical iterative synthesis has a tremendous potential. Noteworthy, the listed libraries made in a one-by-one manner are remarkably smaller than libraries made in

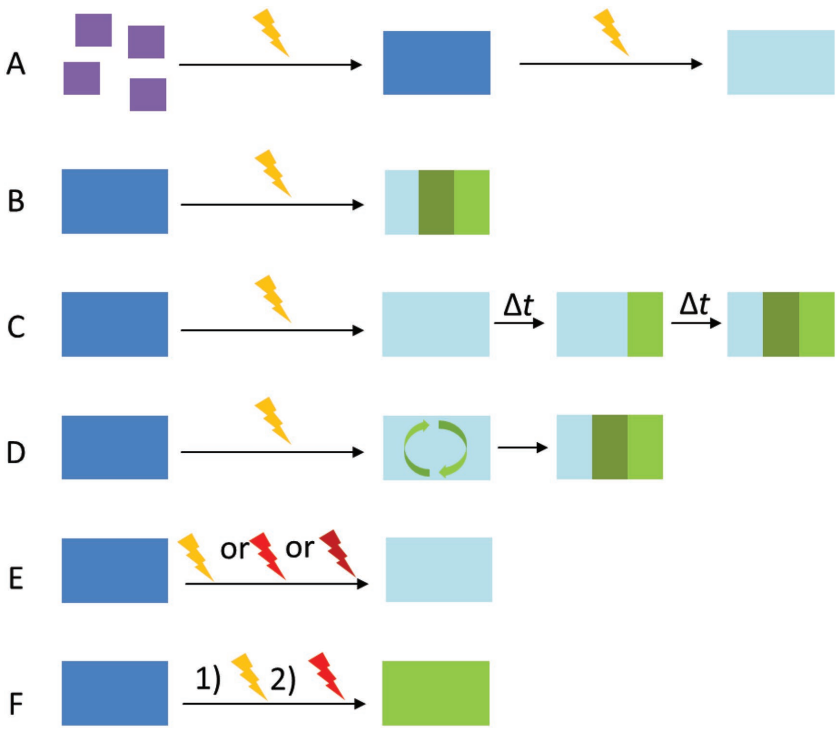


**Figure 5.** Combinatorial high-throughput methods can accelerate the search for materials with complex stimuli-responsive patterns. Examples of possible patterns are described. A) External stimulus is used to first synthesize the material and then trigger the response (e g., photopolymerizable and photodegradable hydrogels); B) one stimulus triggers several responses (simplifying the application of the material); C) multiple responses are orthogonal (independent from each other) and can occur simultaneously or delayed from each other in time (the time and chronological order of responses can be fine-tuned). D) Multiple responses are coupled (upon trigger, a material responds in a certain manner, which causes other responses via the domino principle). E) Multiple stimuli trigger one response (allows more flexibility in application); F) Chronologically first stimulus can switch on/off or fine-tune the response upon second stimulus (as, e.g., presence of ions, redox factors, and light can trigger next response, e.g., thermoresponsiveness).[36]

form of polymer microarrays and still much bigger than conventionally achievable in iterative one-by-one synthesis, making the identification of "hit" materials more likely. An arrayed format, employed in some studies, makes decoding of the "hit" composition simple and is particularly suitable for HT synthesis, subsequent biological screening, and powerful, surface-sensitive analytical methods such as ToF-SIMS and AFM.

To supply the demand for tailored responsive materials that are fine-tuned for a specific application, we should consider using combinatorial HT methodologies to develop materials with customized numbers and sequences of stimuli and responses or with complex stimulus/response patterns (**Figure 5**).[36] For example, a wide range of stimuli should be exploited for drug-delivery applications to trigger drug release under precise spatial and temporal control.[37] Such materials are often not designed rationally, so combinatorial HT screening over random libraries might play an ever bigger role in their development. The optimization of "learning-trained" materials that alter their response after the execution of external stimuli several times could also be accelerated via combinatorial HT methods

We believe that accelerating material discovery will lead to new types of responsive materials triggered by "nonclassical," underutilized stimuli, for example different tastes[38] and smells, haptics (e.g., rough and smooth surfaces), confined spaces (e.g., injectable hydrogels react differently to different pore size), neural impulses, acoustic stimuli (human voice, car noise, or ring of a phone), or gravitational force. Neural impulses, as an example, might be used in different types of actuators. The sensitivity and differentiation of the intensity and duration of stimuli could be improved and refined (e.g., as some light responsive materials can differentiate various wavelengths to release the appropriate cargo,[39] a material responsive to a specific mechanical force should be able to distinguish between a hard punch and soft touch, between a rhythmic and arrhythmic heartbeat). New responses could be introduced, e.g., a response in human and machine-readable format (barcode, numerals, signs, and letters).

By employing combinatorial HT synthesis and screening, we envision the emergence of materials that tangentially attain the complexity of natural dynamic systems. Given the complex interplay of multiple stimuli in vivo, methods for the in vivo testing of such combinatorial libraries of responsive materials should also be further explored.[40]

HT combinatorial methodologies have proven to be powerful techniques in small molecule and material synthesis. We see an immense amount of untapped potential in using HT combinatorial synthesis and screening in the field of research and development and customization of responsive materials, and hope to benefit more from its applications in the very near future.

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## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

combinatorial libraries, high-throughput synthesis, material microarrays, smart materials, stimuli-responsive

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