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COMBINATORIAL AND RATIONAL APPROACHES TO POLYMER SYNTHESIS FOR MEDICINE

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Abstract

High-throughput, combinatorial methods have revolutionized small molecule synthesis and drug discovery. By combining automation, miniaturization, and parallel synthesis techniques, large collections of new compounds have been synthesized and screened. It is becoming increasingly clear that these same approaches can also assist the discovery and development of novel biomaterials for medicine. This review examines combinatorial and rational polymer synthesis for medical applications, including stem cell engineering and nucleic acid drug delivery.

Keywords

Combinatorial; polymer; synthesis; stem cells; nucleic acid delivery; rational design; drug delivery

INTRODUCTION

Polymers are an important class of materials for a broad array of medical applications, including tissue engineering [1] and drug delivery [2]. Because of the complexity of biological systems, the definition of optimal biomaterial design criteria can be difficult, and consequently the discovery of desirable materials through low-throughput, iterative design can be challenging [3]. In the absence of clear design criteria, a high-throughput, combinatorial approach can be beneficial. High-throughput, combinatorial approaches afford several advantages, including decreased costs and cycle times as well as increased probability of discovering a material with desired properties fortuitously [4]. This strategy has transformed drug discovery in the pharmaceutical industry [5] and, more recently, has been applied to materials science [6].

Combinatorial endeavors typically incorporate both library synthesis and high-throughput screening, which are generally conducted on a small scale and in an automated manner [7]. Depending on the ultimate application of the materials of interest, automated assays must often be developed to characterize the constituent members of a given library in order to quickly

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identify promising candidates. Although preliminary library screening commonly imparts general information about trends, iteration is often required to optimize material properties [4].

Combinatorial libraries can enable the synthesis of a collection of polymers that share selected properties while exhibiting incremental variation of other properties. This allows for the isolation of particular compounds that belong to a broad class of materials of interest (*e.g.* biodegradable) that can be functionally and physically discriminated (*e.g.* degradation rate and glass transition temperature). Computational methods have been shown to complement combinatorial synthetic efforts, facilitating the design of polymers with specific properties. Specifically, virtual polymer libraries utilize rational design criteria to interrogate diverse structural space and to accelerate the discovery of desirable materials [8].

Polymers are amenable to modification and afford access to a number of different chemistries and material properties. Accordingly, this class of materials can be utilized in many fields. Here, we discuss the use of combinatorial approaches to polymer library synthesis with an emphasis on their medical application. We further compare the combinatorial strategy to that of rational design.

COMBINATORIAL POLYARYLATE SYNTHESIS

To create libraries of structurally-related polymers whose material properties vary in a predictable and systematic fashion, Brocchini *et al.* introduced the concept of permutationally-designed monomer systems [3]*. This combinatorial approach involved alternating A–B type copolymers in which the first monomer possessed a reactive group for the conjugation of a series of pendent chains, and the second monomer permits systematic modification in the polymer backbone structure. This early example of a combinatorial polymer library featured 112 polyarylates, derived from the reaction of 14 tyrosine-derived diphenols with eight aliphatic diacids (see Figure 1). This study increased the throughput of analysis of a class of biomaterials that, based on natural metabolites, is degradable and boasts potential utility in medical implants [9].

Inspection of this library imparted structure-property correlations [10]. The variations of pendant chains and backbone structure yielded observable differences in polymer free volume, bulkiness, flexibility and hydrophobicity. The polymers exhibited a range in glass transition temperature, surface wettability, and cellular response; however, because they were all derived from very similar monomers, they could be synthesized under identical reaction conditions and displayed common material properties.

In addition to the A–B type copolymers just described, copolymers with varying percent molar fraction of the two monomers can be synthesized combinatorially. By reacting two diols, which can be condensed using phosgene, Yu *et al.* were able to vary not only the pendant chain on the tyrosine-derived diphenol but also the percent molar fraction of the PEG component, yielding a library of tyrosine-PEG-derived poly(ether carbonates) [11]. Again, general structure-property relationships could be established. Specifically, the glass transition temperature and tensile modulus decreased with decreasing PEG block length, increasing PEG content, and increasing pendent chain length.

COMBINATORIAL ENZYMATIC POLYMER SYNTHESIS

The use of enzymes as a means to catalyze polymer synthesis has garnered increasing interest because they can be used in mild reaction conditions, ligate substrates that are generally biocompatible and biodegradable, and afford high region-, stereo-, and chemo-selectivity

[12]. Moreover, enzymes provide access to polymeric structures that feature the diversity, complexity, and function of molecules found in nature.

In an early study of combinatorial enzymatic copolymerization, lipase was used to produce ester copolymers from lactones, divinyl esters, and α,ω -glycols (see Figure 2) [13]. This work led to the observation of two different modes of polymerization – ring-opening polymerization and polycondensation – occurring simultaneously in a single pot, apparently via the same acyl-lipase intermediate.

Polyesters can also be derived from sugars. Park *et al.* exploited the fact that a combinatorial approach can also be used to identify a suitable enzymatic catalyst for a specific reaction [14]. The authors screened commercially-available proteases and lipases for their ability to acylate regioselectively sucrose and trehalose with divinyladipic acid ester. Enzymes with the desired substrate tolerance and activity were identified. The resultant site-specifically-modified diester monomers were reacted with various aliphatic and aromatic diols in the presence of an enzyme to yield linear polyesters with higher molecular weights than those obtained from one-step enzymatic polyester syntheses. Notably, because these polymers comprise sugars, diacids, and diols, the polymers themselves can be produced in a combinatorial manner.

This work was extended accordingly by Kim *et al.*, who synthesized a polymer library in 96-well plates using AA-BB polycondensations of acyl donor and acceptors [12]. Specifically, aliphatic diesters were reacted with carbohydrates, nucleic acids, or diols – aliphatic, aromatic, or a natural steroid – in the presence of lipase. The resultant monomers, which display a remarkable range of naturally-occurring chemical classes with robust chemical and structural complexity, were polymerized in an array format that is readily-amenable to screening.

COMBINATORIAL DEVELOPMENT OF POLYMERS FOR STEM CELL ENGINEERING

High-throughput, combinatorial synthesis schemes also require the development of equally high-throughput characterization and screening systems to allow for the identification of those biomaterials with useful properties. One particularly challenging problem has been the development of biomaterials for use as cellular substrates.

An early high-throughput assay of cell-biomaterial interactions was conducted by Meredith *et al.*, who developed composition spread and temperature gradient techniques for use in screening biomaterials (see Figure 3) [15]*. This method allows for the synthesis of libraries containing hundreds to thousands of distinct chemistries, microstructures, and roughnesses, increasing discovery rate and decreasing variance. The ability of blends of poly(D,L-lactide) and poly(ϵ -caprolactone) to control osteoblast cell function was investigated. Surface features manufactured from particular compositions and under specific processing conditions that significantly improved alkaline phosphatase expression were revealed.

Stem cells possess remarkable potential for use in tissue engineering and regenerative medicine [16,17]. In addition to their unique ability to undergo nearly-unlimited self-renewal in an undifferentiated form [18], human embryonic stem cells boast the potential to differentiate into any cell type in the human body [19]. Importantly, stem cell behavior and differentiation are modulated by interaction between the cells and the surfaces on which they grow.

To repair, sustain, and improve tissues and organs *in vivo*, scientists may employ bioactive degradable scaffolds to control the expansion and differentiation of stem cells [1,20]. Several biomaterials have been utilized to regulate a variety of cell behaviors for tissue engineering applications, including adhesion, proliferation, and differentiation [21–24]. Automated, high-

throughput methods for synthesizing and screening combinatorial biomaterial libraries and cellular microenvironments will accelerate the discovery of factors that control stem cell behavior [25].

To identify biomaterials with differential stem cell attachment, expansion, and differentiation properties, a microarrayed, combinatorial library of polymeric surfaces was synthesized [26]*. The acrylate, diacrylate, dimethacrylate, and triacrylate monomers were spotted in various ratios onto a layer of poly(hydroxyethyl methacrylate) covering an epoxy-coated slide and subsequently photopolymerized. 1,728 individual polymer spots could be arrayed onto a single conventional glass slide, and 20 such slides could be produced in a single day; this throughput enabled the researchers to discover materials that permit differential levels of cell attachment, cell spreading, and cell-type specific growth. This platform is also amenable to the inclusion of soluble growth factors, which are also known to control stem cell behavior [27].

This methodology was extended to include materials synthesized prior to spotting on the microarray and was applied to a library of polyesters [28]. Specifically, 3,456 blends of biodegradable polymers – primarily various ratios of poly(lactide-*co*-glycolide) – on a single slide were screened separately for their interactions with human mesenchymal stem cells, neural stem cells, and primary articular chondrocytes.

COMBINATORIAL POLYMERS FOR NUCLEIC ACID DELIVERY

In addition to influencing cell behaviors, combinatorial polymer libraries can be used to deliver therapeutic molecules to cells, and in some cases *inside* of cells. The development of safe and effective gene delivery systems has proven challenging. Nucleic acid delivery can be facilitated by both viral and non-viral vectors. Viruses have evolved for this purpose and, accordingly, make effective gene delivery systems. However, virus-mediated delivery poses safety concerns, including acute toxicity and the induction of cellular and humoral immune responses [29].

There are a number of potential advantages to non-viral delivery systems, including safety, cost, ease of manufacture, and nucleic acid carrying capacity. Examples of non-viral nucleic acid delivery include physical manipulation of naked plasmid deoxyribonucleic acid (DNA), using techniques such as electroporation, magnetofection, and ultrasound [30]. Additionally, synthetic vectors have been created to improve bio-stability and delivery. Polycationic lipids [31,32] and polycationic polymers [33,34] can electrostatically bind and condense nucleic acids to form nanometer-sized complexes, which are capable of facilitating cellular uptake [35].

Early DNA delivery polymers, such as poly(ethylene imine) (PEI) and poly-L-lysine, can be toxic, in part due to the poor biocompatibility of nondegradable materials [36,37]. A combinatorial approach to this problem was developed around degradable poly(β -amino esters). Polymerization proceeds via the Michael-type conjugate addition of a primary or secondary amine to a diacrylate (see Figure 4) [38]*. This synthetic scheme has several advantages: there are a number of commercially-available starting reagents; no by-products or side reactions are generated; and no catalysts, purification, protection or deprotection chemistry is required. These simplicities render the platform amenable to combinatorial library synthesis in a high-throughput manner.

A preliminary library based on the combinatorial reaction of seven diacrylates and twenty amines was synthesized [39]. The amines were selected to incorporate diverse chemical functionalities such as alcohols, ethers, and amines as pendant chains. The polymers' molecular weights were determined by gel permeation chromatography, DNA-binding abilities were determined by gel electrophoresis, and transfection abilities were determined using a luciferase-encoding reporter plasmid. Two materials (B14 and G5) that induced luciferase

expression levels 4–8 times higher than the positive control (PEI) were identified in the initial screening process.

The resultant materials could be readily characterized to correlate differences in polymer structure with differences in polymer function, thereby facilitating the rational design of future nonviral vectors for gene delivery. Akinc *et al.* investigated the contribution of cellular uptake, particle size, zeta potential, and the pH environment of delivered DNA to effective transfection [40]. They found that, in general, small particle sizes and positive surface charges enhanced cellular uptake *in vitro* and that these attributes were often associated with polymers possessing multiple amines per repeat unit. They also discovered that polymers featuring an imidazole group or two amines in close proximity could successfully avoid low-pH lysosomes. Finally, polymers comprising less hydrophobic diacrylates were typically less cytotoxic.

Akin *et al.* further examined the importance of polymer molecular weight, polymer chain end-group, and polymer/DNA ratio to transfection ability [41]. The members of the combinatorial poly(β -amino esters) library were characterized by NMR, gel permeation chromatography, gel electrophoresis, luciferase and green fluorescent protein transfection, cytotoxicity assay, cellular uptake experiments, and polymer-DNA binding titration.

The high-throughput, combinatorial development of polymers is particularly challenging because of the viscosity and solubility issues associated with higher-molecular weight materials. For DNA delivery, polymers must be processed after synthesis to form nanoparticles with DNA before application to cells, thus necessitating additional steps. In order to expand throughput, Anderson *et al.* designed a semi-automated process that integrated synthesis and screening [42]. Dimethyl sulfoxide was identified as a relatively non-toxic solvent that would enable the viscous monomers and solid polymers – which are not readily manipulated on the small scale – to be retained in the liquid phase. Semi-automated methods were developed to formulate nanoparticles in parallel for direct testing of DNA delivery to mammalian cells without need for purification. 2,350 structurally-unique, degradable cationic polymers were screened, of which 46 were found to be superior to the standard polymeric gene delivery agent PEI.

A next-generation library of over 500 members was synthesized – controlling for molecular weight and polymer end group (amine versus acrylate) – and screened *in vitro* for the ability to transfect mammalian cells [43]. Following intratumoral injection, the top-performing polymer, afforded four-fold better DNA delivery of a plasmid encoding the reporter protein luciferase than jetPEI, one of the premier commercially-available DNA delivery polymers. C32 was subsequently used to deliver a construct that encoded the lethal A chain of diphtheria toxin to xenografts derived from LNCaP human prostate cancer cells, resulting in inhibition of tumor growth as well as regression in 40% of the tumors. Significantly, a convergence of structure was observed: the top three polymers differed by only one carbon atom in the repeating unit and featured a terminal hydroxyl group pendant from the backbone amine.

Green *et al.* expanded the application of poly(β -amino esters) to deliver DNA to primary human endothelial cells [44], which are much harder to transfect than cancer cell lines. A combinatorial library of diacrylates and amines that bore great structural similarity to the precursors C and 32 was synthesized to achieve a range of molecular weights. The members were screened at various polymer:DNA ratios, and biophysical properties such as particle size, ζ -potential, and particle stability were evaluated over time. These properties changed markedly in the presence of serum proteins, and the properties of these serum-interacting particles were shown to correlate with transfection efficacy, which exceeded that obtained using the premier commercially-available reagents. The data indicate that poly(β -amino esters) may be useful vectors for gene therapy applications in cardiovascular disease or cancer.

An additional round of polymer optimization was achieved by combinatorial modification of the polymer termini [45]. Optimizing reaction conditions so that the polymers could be synthesized rapidly and screened without needing to be purified, the authors examined the effect of many structurally-diverse terminal amines. The synthesis of a library of end-modified C32 polymers yielded several members with greatly improved transfection ability both *in vitro* and *in vivo*.

Other innovative combinatorial approaches to the synthesis of polymeric carriers for applications in nucleic acid delivery have been explored. Murphy *et al.* synthesized a combinatorial library of cationic N-substituted glycine oligomers with varying length, charge density, side-chain shape, and hydrophobicity [46]. A 36-mer containing 12 aminoethyl side chains was identified as the most efficacious member of the library, enabling the transfection of multiple cell lines with efficiencies comparable to or greater than the premier commercially-available reagents. This class of polymers is both protease resistance and serum insensitive. Additionally, the underlying chemistry is less expensive than traditional peptide synthesis, affords modular construction, and provides access to diverse side-chain functionalities.

Most recently, Thomas *et al.* synthesized a combinatorial library of 144 biodegradable derivatives of two small PEIs – one linear and one branched – and 24 bi- and oligo-acrylate esters [47]. The top-performing and least toxic polymers *in vitro* were screened *in vivo* via systemic administration to mice. The leading candidates readily outperformed linear 22-kDa PEI, which is considered to be one of the foremost gene delivery vectors both *in vitro* and *in vivo*, and were also found to be markedly less toxic. These cross-linked, biodegradable PEIs appear to be optimal vehicles for nucleic acid delivery to the lung, in particular.

RATIONALLY-DESIGNED MATERIALS FOR DRUG DELIVERY

Successes in material design have not been limited to combinatorial approaches. There are many examples of rationally-designed systems that have made important contributions to the field of drug delivery. Below, we describe some examples of these systems and contrast them with the combinatorial methods described above.

Dendrimers feature repeating units of specific structural entities and are therefore closely related to linear polymers. However, whereas many polymers can be synthesized in one synthetic step, dendrimers are produced via a series of reactions that connect the subunits to a central core. Each successive generation builds on the existing scaffold, resulting in controlled molecular weight, shape, and size. Since their initial report, the potential application of dendrimers has been explored in many contexts [48,49]*. Here, we will focus more on the criteria for designing dendrimers for particular applications.

Poly(amidoamine) dendrimers (PAMAMs) are one of the most widely-utilized dendrimers for the development of new therapeutics. The synthesis of these dendrimers is straightforward, and many generations are now commercially available. Consisting of an ethylenediamine (EDA) core linked to EDA arms, this dendrimer has a number of interior tertiary amines and functionalizable primary amines at the surface. Because of these features, PAMAMs have been of particular interest to the gene therapy community as nonviral delivery vectors. The overall positive charge allows for electrostatic binding to both DNA and RNA, and the tertiary amines facilitate endosome release once inside the cell [50].

The interactions of these molecules with DNA have been extensively characterized [51–54]. Later generations of dendrimers were found to be more toxic than earlier generations due to the increase in the number of primary amines at the surface [55]. Additionally, transfection efficiency was generation-dependent, with early and late generations less effective than intermediate ones [52]. This finding is attributed to the delicate balance mediated by primary

amines binding DNA and tertiary amines buffering endosomal pH. Although PAMAMs were found to have equivalent transfection to and lower cytotoxicity than either branched or linear PEI, the overall efficiency is still less than that observed using viral vectors [56,57].

The use of dendrimers as RNA transfection agents is currently an active area of research. Dendrimers have been shown to interact with many types of RNA, such as ribozymes, TAR RNA, and total cell RNA [58–60]. Given the potential of RNAi-based therapeutics and the similar characteristics of RNA to DNA, there is widespread interest in using dendrimers as RNA transfection agents. While there are few reports of unmodified PAMAMs having success in this capacity [61]. Modification of the terminal amines with various functional groups has shown some promise. While the conjugation of TAT peptides was not efficacious, a recent report suggests that cyclodextrin-conjugated PAMAM can be successfully utilized for siRNA delivery [62,63]. Baigude *et al.* describe a system termed interfering nanoparticles (iNOPs) that features more dramatic modifications to the PAMAM dendrimer [64]. In this report, the ligation of lysine residues and lipid chains to the terminal amines yielded a molecule that was more efficient than Lipofectamine for siRNA-induced silencing of the ApoB gene and decreased cholesterol levels *in vivo*.

Covalent modification of the termini appears to be the next step forward in producing viable dendrimer-based therapies. The ligation of PEG to dendrimers has been used to increase serum stability [65–67]. Additionally, the free termini have been capped to shield the primary amines in an effort to reduce toxicity. Targeting ligands such as transferrin, galactose and mannose have been appended to increase gene delivery to specific organs or cell types [68,69]. Other functional groups, such as guanidinium and alkyl chains, have been shown to increase the delivery efficiency of DNA [70–72].

While these approaches are all efforts to improve the utility of dendrimers as nonviral vectors for gene therapy, there is significant interest in other therapeutic realms. In addition to their use as transfection agents, there has been substantial progress in the development of dendrimers as delivery agents for chemotherapeutics. Anticancer drugs such as paclitaxel, cisplatin, doxorubicin, and 5-aminolaevulinic acid have been attached to dendrimers to achieve successful transport across the cell membrane [73–76]. Folic acid targeting to tumors has also been achieved [77,78]. The conjugation of fluorescent dyes or nanoparticles highlights the potential of dendrimers as components of new imaging agents [79,80].

Dendrimers have significant potential for use as therapeutics. As transfection agents for gene therapy, they yield similar efficiency to the industry standard polyethyleneimine (PEI) with decreased toxicity but do not match the efficiency of viral vectors [81]. While synthetic modifications for use as chemotherapeutics and imaging agents remains complex, perhaps greater potential lies in these fields, where the delivery of DNA inside the nucleus of a cell is not required.

Dynamic PolyConjugates represent another class of carriers for siRNA delivery that was recently described [82]. This system features an amphipathic poly(vinyl ether) conjugated to siRNA, PEG and the targeting ligand N-acetylgalactosamine via reversible linkages. The complex is designed to target specific hepatocytes, enter the cell through endocytosis and trigger endosome release through endosmosis. Upon release, the disulfide linkage to the siRNA is cleaved by the reducing intracellular environment. Efficient knockdown of ApoB was shown, demonstrating the potential of rationally-designed polymeric delivery systems for therapeutic RNA delivery.

Other rationally-designed systems for the delivery of genetic therapies are found in the realm of cationic lipids. These amphiphiles feature a positively charged head, a linker, and long alkyl chain tails that, in the presence of the negatively-charged nucleic acids, spontaneously assemble

into lipoplexes [83]. Beginning with reports by Felgner and coworkers, cationic lipids have been investigated for the cellular delivery of both DNA and RNA, and reviews of their use in this capacity abound in the literature [84,85]*.

As with dendrimers, there is significant success observed with cationic lipids and dynamic polyconjugates. However, this approach is limited by the difficult synthetic modifications that must be made to achieve diversity within the structure set. As none of these approaches have yielded a nonviral vector of comparable efficiency to viral vectors, new structure spaces needs to be examined. A report of structurally-unique cationic lipids isolated from a library of compounds synthesized on solid phase may be a prelude to new techniques for nonviral vector discovery [86]. Combinatorial approaches to surveying this space may provide the greatest chance to discover new molecules for the delivery of therapeutics across the cell membrane and lead to a new era in drug delivery.

CONCLUSION

Rational-design approaches have resulted in important advances in polymers for drug delivery and other medical applications. More recently, the advancement of combinatorial synthetic methods and high-throughput screening procedures has significantly increased the pace of discovery of new polymers. Unlike combinatorial approaches used with small molecules, high-throughput combinatorial development of polymers has proven challenging, in part because of the physical properties of polymers. However, a growing collection of automated polymer synthesis and manipulation technologies have been developed, resulting in a variety of novel biomaterials. Combinatorial approaches to polymer synthesis and analysis have resulted in comparative data, enabling the elucidation of structure-activity relationships. The computational modeling of these growing sets of data is further accelerating the pace of new polymer discovery and optimization. In our opinion, the combination of rational, combinatorial, and computational methods will provide a powerful tool set for the future design of polymers for medicine.

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Figure 1.

Polyarylates were synthesized by the condensation of tyrosine-derived diphenols and aliphatic diacids. Reproduced pending permission from [3].

Figure 2. Enzymatic copolymerization of lactones, divinyl esters, and glycols was performed using lipase as catalyst to produce ester copolymers. Reproduced pending permission from [13].

Figure 3.

A schematic of the continuous composition gradient deposition process. Reproduced pending permission from [15].

Figure 4.

Poly(β -amino ester)s were synthesized by the conjugate addition of primary or bis(secondary amines) to diacrylates. Reproduced with permission from [42].