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Bioreducible Polymers for Gene Silencing and Delivery

SEJIN SON, RAN NAMGUNG, JIHOON KIM, KAUSHIK SINGHA, AND WON JONG KIM*

Department of Chemistry, Pohang University of Science and Technology, Pohang 790-784, Korea

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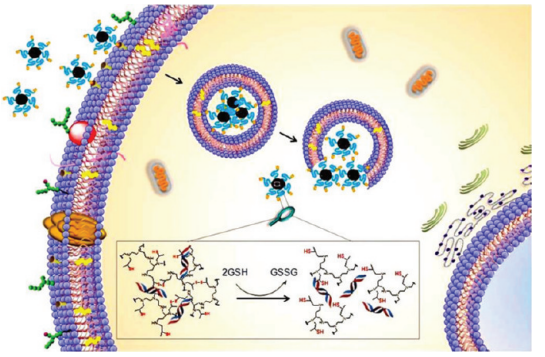
CONSPECTUS

Polymeric gene delivery vectors show great potential for the construction of the ideal gene delivery system. These systems harness their ability to incorporate versatile functional traits to overcome most impediments encountered in gene delivery: from the initial complexation to their target-specific release of the therapeutic nucleic acids at the cytosol. Among the numerous multifunctional polymers that have been designed and evaluated as gene delivery vectors, polymers with redox-sensitive (or bioreducible) functional domains have gained great attention in terms of their structural and functional traits. The redox environment plays a pivotal role in sustaining cellular homeostasis and natural redox potential gradients exist between extra- and intracellular space and between the exterior and interior of subcellular organelles. In some cases, researchers have designed the polymeric delivery vectors to exploit these gradients. For example, researchers have taken advantage of the high redox potential gradient between oxidizing extracellular space and the reducing environment of cytosolic compartments by integrating disulfide bonds into the polymer structure. Such polymers retain their cargo in the extracellular space but selectively release the therapeutic nucleic acids in the reducing space within the cytosol. Furthermore, bioreducible polymers form stable complex with nucleic acids, and researchers can fabricate these structures to impart several important features such as site-, timing-, and duration period-specific gene expression. Additionally, the introduction of disulfide bonds within these polymers promotes their biodegradability and limits their cytotoxicity.



Many approaches have demonstrated the versatility of bioreducible gene delivery, but the underlying biological rationale of these systems remains poorly understood. The process of disulfide reduction depends on multiple variables in the cellular redox environment. Therefore, the quest to unravel various issues such as the site and time of disulfide bond reduction during the cellular uptake and trafficking have stimulated a number of interesting studies which have employed disulfide compounds with a variety of reducible linkers. Such studies help researchers understand not only how modifications made to disulfides can alter their thiol–disulfide exchange characteristics but also to decipher the effect of the induced changes on the dynamics of the redox environment.

This Account discusses current research trends and recent progress in the disulfide chemistry enabling novel and versatile designs of reducible polymeric gene delivery systems. We present strategies for the introduction of disulfide bonds into polymers. These representative examples and their respective outcomes elaborate the benefit and efficiency of disulfides at the individual stages of gene delivery.

Introduction

The immense potential of gene therapy in influencing gene expression to induce therapeutic cellular processes and responses could actually be translated into successful clinical applications if the diverse and often interconflicting impediments associated with the whole process can be addressed with utmost ingenuity and alertness. Since its emergence, polymeric vector-mediated gene therapy,

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which is preferred over viral vectors owing to the associated toxicity and immunogenicity concerns,[1] has come through a significant evolution encompassing improved loading capacity, greater safety measures, and high tunability to integrate multiple functional traits to surmount various impediments.[2] However, there is ample scope for further refinements and advancements in each of the key steps such as condensation of nucleic acids, stability in physiological conditions, transportation to specific target cells, cellular uptake, and release of therapeutic payloads.[3]

The rational design of polymeric vectors must render coexistence of two interconflicting attributes, namely, condensation and release of nucleic acids. Cationic polymers possess the abilities to form compact polymer complex (polyplex) with nucleic acids and to trigger efficient cell internalization and endosomal escape; however, such strong complexation impedes the release of nucleic acids and retards the dissociation of polyplexes at the cytoplasm entailing a major hurdle to initiate transcription and enhance gene expression.

The common strategy adopted to integrate contrasting features to the same vector involves integration of stimuli-responsive attributes to the fabricated vector systems. Different intracellular and extracellular domains provide diverse physiological environments which differ with respect to pH, temperature, redox gradients, osmotic pressure and enzymes. Exploitation of redox gradient within the intracellular and extracellular region can address both the conflicting issues of condensation and release of nucleic acids. Bioreducible polymers having disulfide linkages can form nanosized polyplexes which are sufficiently stable during the circulation and in the extracellular region, however undergo rapid cleavage under a reductive environment through glutathion (GSH)-mediated thiol-disulfide exchange reactions (Figure 1). The quick responsive chemical degradation kinetics of bioreducible polymers is advantageous over commonly used hydrolytically degradable polymers such as aliphatic polyesters and polycarbonates which demonstrate significantly slow gradual degradation kinetics.[4]

Apart from accommodating the two contrasting traits, disulfide bonds play a crucial role in suppressing the toxicity arising due to the accumulation of the high molecular weight (HMW) cationic vectors. Such cationic polymers cannot be metabolized or broken down by cellular enzymes, however HMW transfection complexes having disulfide bonds disintegrates into the parent low molecular weight (LMW) fragments which should undergo facile excretion from the body without inducing significant cytotoxicity (Figure 1).

This Account will provide an insightful account of the underlying principles and rationale which govern the design and development of bioreducible polymers in eliciting highly efficient gene delivery. This article will also narrate the evolution of multifunctional and structurally diversified bioreducible polymeric vectors in gene delivery citing various examples covering the delivery of therapeutic payloads to the target organs.

Redox Environment in Biological System

Redox environment indicates the biological status of the cell which is composed of many redox couples such as nicotinamide adenine dinucleotide phosphate ($NADPH/NADP^+$), thioredoxin (TRX_{red}/TRX_{ox}), and glutathione (GSH/GSSG), each having different reduction potential and reducing capacity.[5] The regulation of redox potential governs the meta-/catabolism and redistribution of cellular energy and is crucial in maintaining cellular homeostasis.[6,7] GSH which consists of serine, cysteine, and glutamate imparts a more pronounced effect in regulating the redox potential in and out of cells primarily owing to its high concentration amounting approximately 500- to 1000-fold higher than TRX and NADPH.[7,8]

In all cell types gamma-glutamylcysteine synthetase (γ-GCS) and GSH synthetase catalyze the synthesis of GSH from L-glutamate. Subsequently, GSH is delivered to specific intracellular compartments and extracellular spaces though mechanistic investigations of GSH transport into the intracellular organs have not been reported. GSH plays an important role as antioxidants, and in regulating the protein structure and function, cell signaling, proliferation and apoptosis.[9]

GSH-induced redox-potential gradient between the extra- and intracellular space significantly governs the outcome of gene delivery. The concentration of GSH in intracellular compartments (3−10 mM) is maintained approximately 3 orders of magnitude higher than that in extracellular plasma ($\sim$2.8 μM) (Figure 1).[10,11] This large difference in GSH concentration has been exploited in achieving highly efficient redox-responsive gene delivery. Low GSH concentration provides a high stability of the delivery system outside cells, while high concentration of GSH triggers rapid release of nucleic acid from the delivery system by cleavage of disulfide bond in the intracellular space.[4]

Bioreducible Polyethylenimine (PEI)

Nonviral vectors based on polyethylenimine (PEI) and derivatives have gained enormous attention in gene therapy.[12]

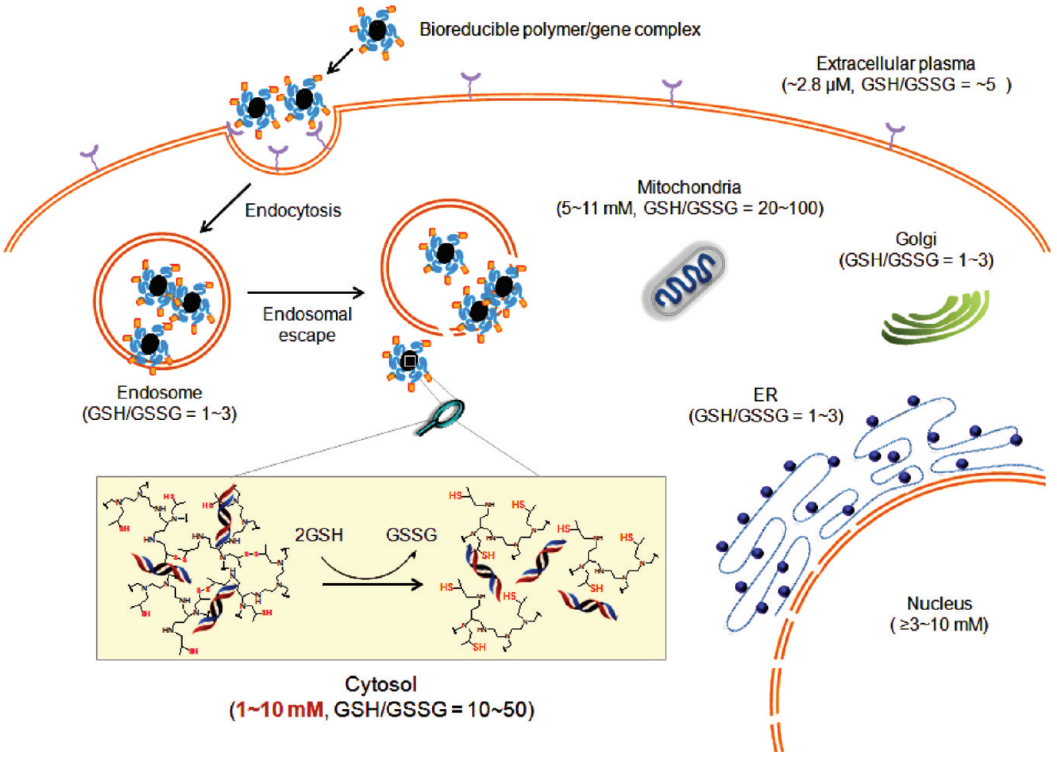

FIGURE 1. Schematic illustrations of the gene delivery by bioreducible polymeric vector. Bioreducible polymers and DNA form nanosized polyplexes which remain stable during circulation and within the extracellular region. GSH-mediated thiol-disulfide exchange reactions trigger rapid dissociation of polymer under the reductive environment.

PEI has been widely used in nonviral transfection in vitro and in vivo, and gains advantage over other polycations for the strong DNA condensation ability and the intrinsic endosomolytic activity stemming from its proton sponge effect.[13,14] HMW PEI, by forming stable polyplexes, can effectively protect DNA against degradation by lysosomal enzymes. However, high gene delivery efficiency of HMW PEI also induces greater cytotoxicity, and furthermore, the strong packing of DNA in HMW PEI/DNA polyplexes becomes a critical hurdle during the release of DNA inside the cytoplasm. The common strategy adopted so far to overcome the trade-off between transfection and toxicity involves the conversion of LMW PEI into the HMW vector through cross-linking with biodegradable linkages such as esters, amides, orthoesters, acetals, glycosides, and disulfides.[15] Incorporation of disulfide linkage in PEI has drawn immense attention in recent time, as it not only addresses the cytotoxicity concerns but also provides an excellent means to release the DNA into the cytosol in presence of GSH. The general strategy to introduce disulfide linkages into the polymeric framework involves two distinct approaches. The prevalent method utilizes cross-linkers with disulfide moieties during the construction of delivery vector, and the other approach involves prethiolation which features installation of thiol group to PEIs and subsequent oxidation of thiolated PEIs (PEI-SH) to cross-linked polymers.

Prethiolation can be accomplished by introduction of the thiol moieties to LMW branched PEI (BPEI800, M_w: 800 Da) utilizing ring-opening reaction of methylthiirane (Figure 2A).[16] The degree of thiolation can be controlled by adjusting the PEI/methylthiirane ratio. Oxidation of the PEI-SH with DMSO affords disulfide cross-linked PEIs (PEI-SS). In vitro experiments showed that the PEI-SS series demonstrated a lower cytotoxicity and higher gene transfection efficiency than HMW PEI with M_w of 25 kDa (PEI25K), a gold standard in polymeric gene delivery agents. However, the disulfide content and M_w of raw PEI influence the in vitro gene delivery efficiency.[17] LMW PEI800 was reported to be the most efficient among the three raw PEIs of different M_w investigated (M_w: 800 Da, 1.8 kDa and 25 kDa). Moreover, the PEIs with too low or too high thiolation degrees are ineffective in forming compact polyplexes resulting in very poor transfection efficiency.

Utilizing prethiolation strategy Kim et al. developed polymeric gene carriers endowed with multifunctional attributes such as tumor targeting, prolonged circulation and bioreducibility by simultaneous installation of various types of functionalities in one-pot reaction under mild conditions. LMW PEI was thiolated with propylene sulfide and mixed with

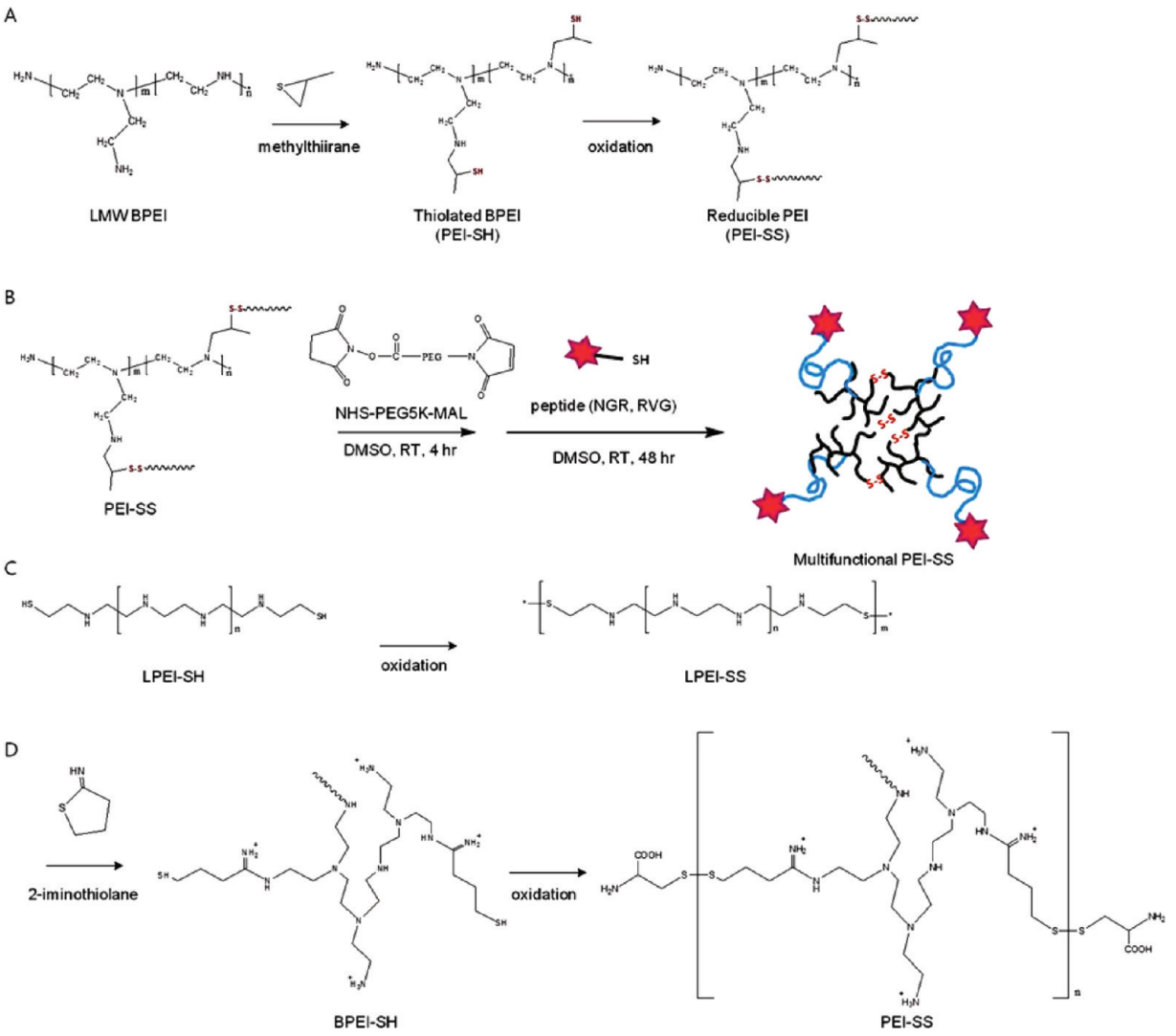

FIGURE 2. Synthetic schemes and chemical structures of various PEI-SS derivatives obtained via functionalization of PEI-SS and prethiolation method.

α-maleimide-ω-*N*-hydroxysuccinimide ester PEG (MAL-PEG-NHS, M_w: 5 kDa) and cyclic NGR (cNGR) peptide (Figure 2B).[18] GSH-mediated reductive cleavage of disulfide linkage triggered efficient release of pDNA leading to efficient transfection in HT1080 cells. Furthermore tumor-specific delivery and GSH-dependent pathway were substantiated by the suppression of transfection efficiency when carried out in the presence of free cNGR and buthionine sulfoximine (BSO) which inhibits the GSH level. The efficacy of such bioreducible delivery system was further demonstrated by the successful delivery of gene to mouse brain overcoming the blood-brain barrier (BBB) through expedient vector construct having RVG peptide as a targeting ligand for neuronal cells (Figure 2B).[19] PEI-SS polymer in combination with RVG peptide and mannitol infusion was successfully employed to deliver neurogenic miRNA into the brain with high efficacy through in vivo neuron-specific targeting.[20]

Park et al. synthesized reducible linear PEI (LPEI-SS) derivatives having disulfide bonds through tosylation of terminal hydroxyl group of ethanolamine derivatives, followed by substitution with thioacetal and deprotection of acetyl and tosylamide groups (Figure 2C). The increase in amine density of LPEI-SS facilitated the increase of transfection efficiency and further it was observed that LPEI-SS with a monomer of 6 to 8 amines showed transfection efficiencies at par with BPEI25K.

The degradation of LPEI-SS in the reductive cytosolic environment of HeLa cells was indirectly confirmed by recovery of fluorescence intensity of LPEI-SS-BODIPY conjugate as the probe–probe quenching effect of BODIPY-FL fluorescence dye was diminished due to the parting of the probes during degradation of polymers.[21]

The prethiolation of polymers was also achieved by employing 2-iminothiolane as thiolating reagent.[22] Bae

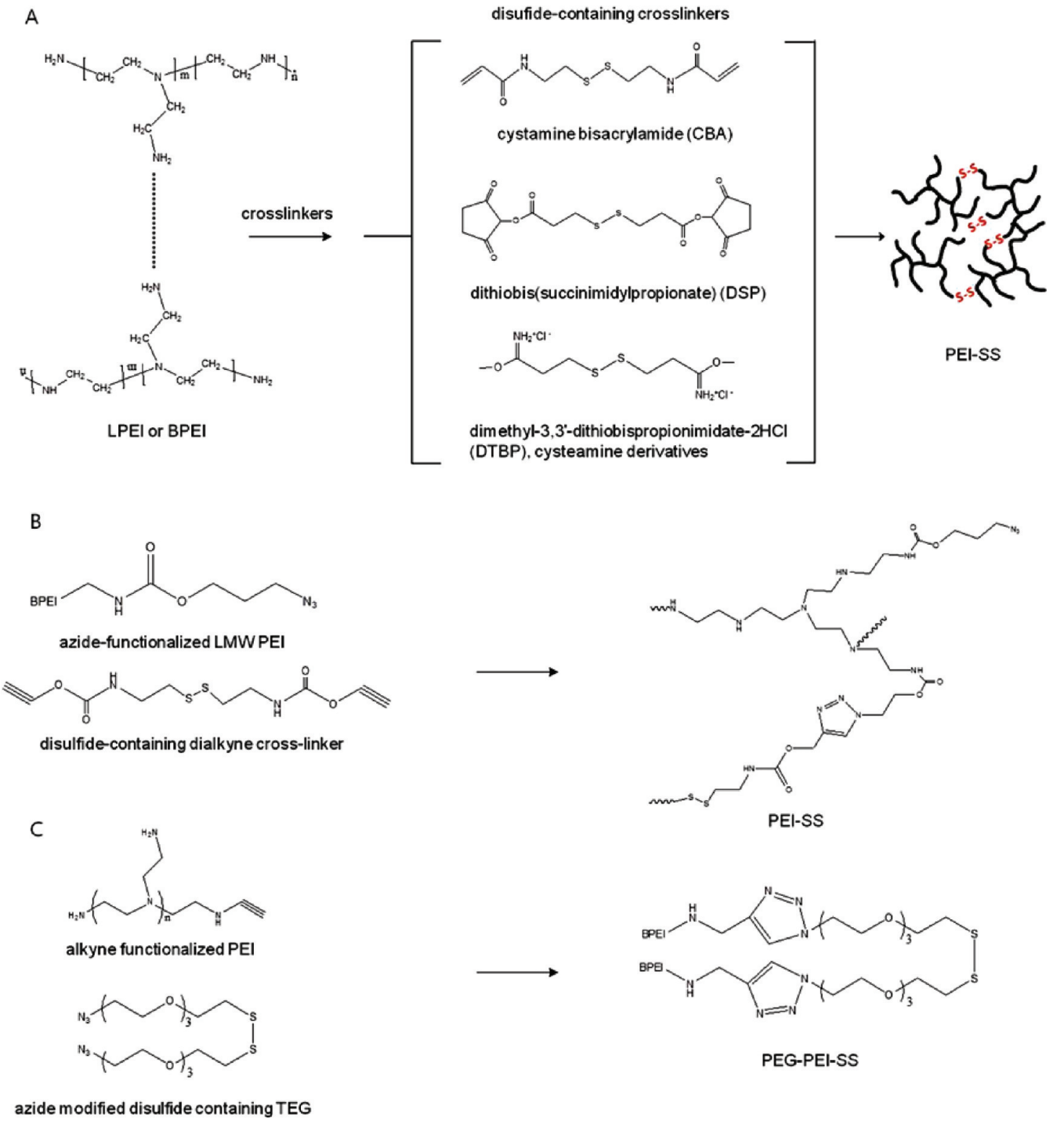

FIGURE 3. Chemical structures of various cross-linkers containing disulfide linkage (A) and utilization of click chemistry for the synthesis of bioreducible PEI-SS (B and C).

and his co-workers synthesized reducible polymers from LMW BPEI800 via 2-iminothiolane-mediated thiolation and subsequent oxidation (Figure 2D). The major advantage of 2-iminothilane lies on the fact that unlike other thiolation reagents such as dithiobis(succinimidyl propionate) (DSP) and *N*,*N*′-cystamine bisacrylamide (CBA), it retains the total number of positive charges maintaining the condensation and buffering ability since 2-iminothilane converts primary amines to amidine groups.

Apart from prethiolation strategies as mentioned above the most common strategy adopted to install bioreducibility to polymers involves the direct conjugation between LMW

polymers with various linkers containing disulfide linkage. Goepferich et al. had synthesized reducible PEI by cross-linking LPEI2.6K (M_w: 2.6 kDa) with cross-linkers such as DSP or boc-cystine to accomplish the polymer degradation in the presence of reducing agents (Figure 3A).[23] Citing an elaborate transfection screening using different cell lines the group demonstrated the superior transfection efficiency and substantially low cytotoxic features of the bioreducible LPEI. Investigations revealed that the structural features of the polymers, for example, branched or linear, affected the physicochemical properties and, hence, transfection efficiency.[24]

The disulfide-containing cross-linked PEI derivatives were also synthesized utilizing click chemistry between disulfide-containing dialkyne cross-linker bis[propargyl carbamate]ethyl disulfide (BPPA-Cyst) and azide-functionalized LMW PEI (Figure 3B).[25,26] In vitro experiments demonstrated that the reducible PEIs not only had much lower cytotoxicity, but also cause superior transfection activity as compared to the control nondegradable BPEI25K. More recently reducible PEI-PEG was synthesized by click chemistry between alkyne-functionalized PEI and azide modified disulfide-containing tetraethylene glycol (TEG) resulting in enhanced transfection efficiency (Figure 3C).[27]

Bioreducible Poly(amido amine) (PAA)

PAAs are peptidomimetic polymers synthesized via Michael-type reaction using varied primary, secondary or tertiary amines and bisacrylate monomers which provides the flexibility in integrating diverse functional attributes under mild reaction condition in the main chain and side chain of the polymers (Figure 4A). Moreover excellent water solubility, relatively low cytotoxicity, and good long-term biodegradability promote PAAs as an interesting candidate in various biomedical applications, including drug and gene delivery as well as tissue engineering.[28]

Engbersen et al. has developed a series of linear reducible PAA (SS-PAA) homo- and copolymers employing Michael addition reaction of secondary and tertiary amine such as 1-(2-aminoethyl)piperazine (AEP) to various bisacrylamide segments (Figure 4A). Varied content of bioreducible disulfide linkages in the main chain have been introduced through disulfide-containing cystaminebisacrylamide (CBA). The SS-PAA polymers exhibited significantly high transfection due to the efficient DNA condensation and effective buffering capacities, and were essentially nontoxic.[29] As a continued effort to modify and refine the SS-PAAs, they installed various pendant groups to the SS-PAAs backbone to regulate their basicity and hydrophobicity, and thereby tuned the DNA condensation, buffer capacity, and transfection efficiency (Figure 4A). The polymers having protonable nitrogen in the pendant groups such as pDMEA and pHIS generally possess high charge density and therefore demonstrate better DNA condensation ability than those lacking protonable nitrogen in the pendant group as in pABOL. Polymers, having low-basicity amino groups ($pK_a < 7$) such as pHIS as well as polymers having hydroxyalkyl side groups such as pABOL and pAPOL, showed higher buffer capacities than BPEI25K, whereas pDMEA, having high-basicity amino groups ($pK_a > 7$), displayed lower buffer capacity. The polyplexes of SS-PAAs comprising HIS, APOL, and ABOL showed high buffer capacities and elicited highest transfection efficiencies. The cell membrane−polyplexes interaction and membrane disruption might influence the transfection efficiency as the observed degree of transfection followed order of the hydrophobic character in the sequence pAPOL > pABOL > pMOPA (Figure 4A).[28,30]

The modified reducible PAAs containing oligoamine side chains (SS-PAOAs) with varied number of amino functions and length of alkyl spacer imparted significant effects on buffer capacity, transfection efficiency, and cytotoxicity (Figure 4A).[31]

Incorporation of hydrophobic characters into SS-PAAs also affects the outcome of transfection efficiency. High degree of benzoylation and acetylation in SS-PAAs with aminobutyl side chains yielded favorable results; however, benzoyl derivatives presumably due to the higher hydrophobic interactions not only formed small and very stable polyplexes but also demonstrated higher transfection efficiencies in COS-7 cells as a result of several collective attributes such as induced increased stabilization of the polyplexes and improved endosomolytic properties and diminished cytotoxic effects arising from the reduction of excessive primary amines in cationic polymers (Figure 4B).[32]

Continued endeavor to explore the feasible and promising structural variation in reducible PAAs also demonstrated the unusual ploy to introduce *N*,*N*′-dimethylcystamine (DMC) as amine segments to variable bisacrylamide monomeric units in these polymers in contrast to using mundane disulfide containing CBA units (Figure 4C). Higher proximity of the disulfide moiety to tertiary nitrogen atoms in the DMC unit led to the lowering of the pK_a value and hence induced high buffer capacity to polymers in the physiological pH range (pH = 7.4−5.1).[33]

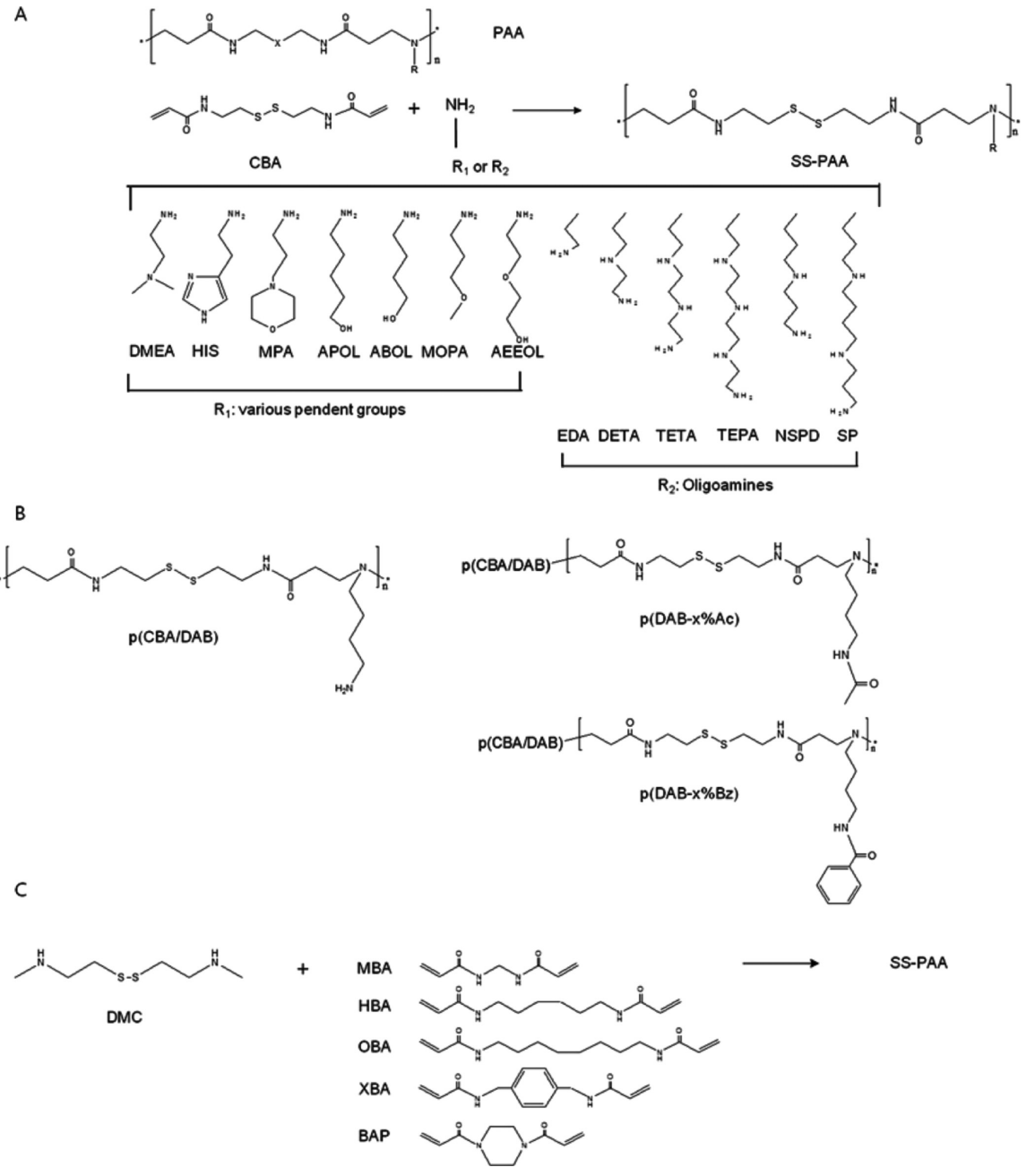

FIGURE 4. Chemical structure and general synthetic route of SS-PAA with different functional groups.

Kim and co-workers reported a newly designed peptidomimetic polymer namely poly(amido ethylenimine) polymers (SS-PAEIs) containing multiple disulfide bonds which could be degraded in intracellular compartment (Figure 5A).[34] They synthesized the SS-PAEIs using three different ethylene amine monomers, that is, ethylenediamine (EDA), diethylenetriamine (DETA), or triethylenetetramine (TETA) and cystamine bisacrylamide (CBA). In vivo application of reducible polymers such as disulfide poly(amidoethylenediamine) (SS-PAED), which were synthesized by addition copolymerization of ethylenediamine (EDA) with cystamine bisacrylamide, demonstrated significant VEGF expression in a rabbit myocardial infarct model by delivering VEGF pDNA, and highlighted the potential for the acceleration of neovascular formation and improvement of tissue function in ischemic myocardium (Figure 5B).[35]

Poly(disulfide amine)s (PDAs) also constitutes an important class of bioreducible polymers containing disulfide bonds, and tertiary amine groups in the polymeric backbone. The transfection efficiencies of PDAs obtained by

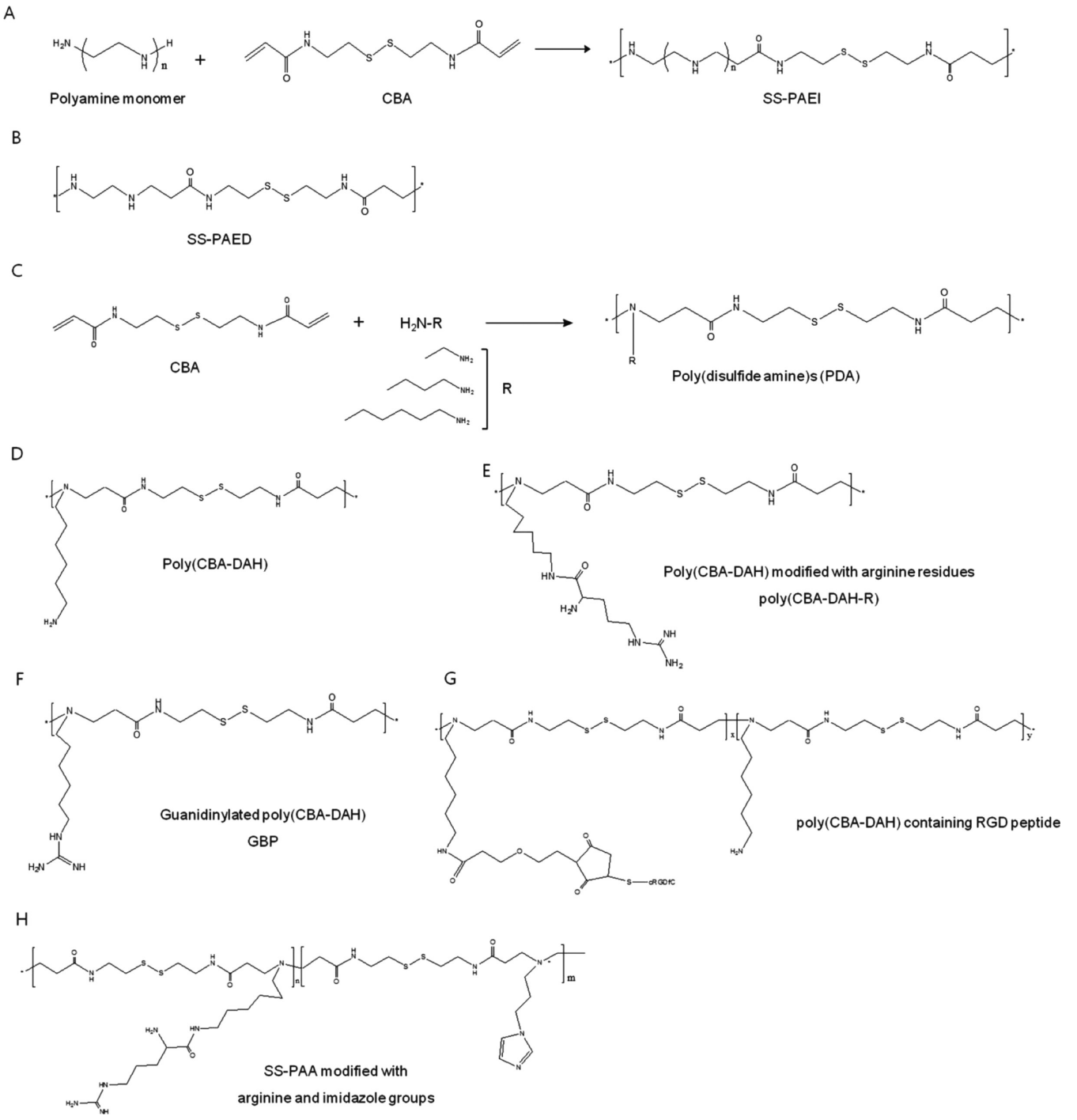

FIGURE 5. Chemical structure and general synthetic route of SS-PAAs derivatives modified with various functional groups.

Michael addition of N-Boc protected diamines (N-Boc-1,2-diaminoethane, N-Boc-1,4-diaminobutane, and N-Boc-1,6-diaminohexane) to CBA and the concomitant deprotection of N-Boc moiety, were influenced by the length of the side-chain spacer appended to these PDAs. The PDA containing hexaethylene spacer, poly(CBA-DAH), exhibited comparable transfection efficiency to BPEI25K (Figure 5C and D).[36] Poly(CBA-DAH) efficiently delivered Fas siRNA into rat cardiomyocytes (H9C2 cells) through PGE_2 receptor-mediated pathway leading to a significant increase in Fas gene silencing and inhibition of cardiomyocyte apoptosis without inducing interferon-alpha in peripheral blood mononuclear cells (Figure 5D).[37] Arginine residues were also integrated into poly(CBA-DAH) to enhance the cellular membrane

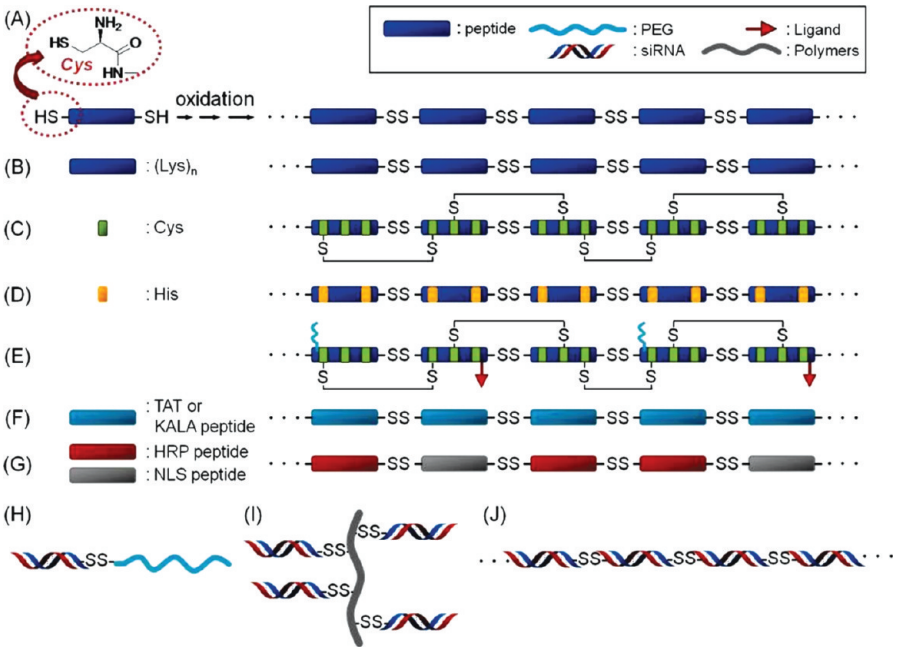

FIGURE 6. Structures and synthetic schemes of various bioreducible polypeptides (A−G) and siRNA conjugates (H−J). (A) General scheme for preparation of bioreducible polypeptides through oxidation of terminal cysteinyl thiol groups of Cys(X)$_n$Cys peptide. (B) Lysine-based bioreducible polypeptides, (C) cross-linked by internal cysteins, (D) containing histidine, (E) cross-linked by internal cysteins and functionalized. (F) Bioreducible polypeptides based on TAT or KALA, (G) based on HRP and NLS peptide. (H) Polymer-ss-siRNA conjugate. (I) siRNA-grafted polymer conjugate with disulfide bond. (J) Disulfide bonds-mediated polymerized siRNA.

however, contrary to the expectations the greatly enhanced transfection efficiency of poly(CBA-DAH-R) did not stem from its high cellular penetrating ability but might be mediated by other factors such as good nuclear localization ability (Figure 5E).[38]

The Kim group has also undertaken several strategies to enhance gene delivery efficiency of the reducible polymer by introducing various functionalities such as guanidine groups meant for enhanced cellular uptake (Figure 5F)[39] and RGD peptide meant for tumor targeting (Figure 5G).[40] Recently, cell penetrating and endosomal buffering attributes were also installed by introducing arginine and 1-(3-aminopropyl) imidazole (API) molecule respectively in poly(CBA-DAH) (Figure 5H).[41]

Peptide-Based Reducible Gene Carrier

LMW peptides have been extensively explored as gene carriers owing to the low side effects and facile synthetic maneuvering which provides the opportunity to optimize the delivery vector to achieve enhanced gene delivery efficiency. The poor gene condensation ability of LMW peptides has been addressed by developing HMW reducible poly peptides (Figure 6A and B). Rice group developed a novel reducibly cross-linked peptide system to impart stability to LMW peptide/DNA complex.[42] LMW peptides obtained by incorporation of one cystein residue to four lysine residues in Cys-Trp-Lys18, condensed pDNA and subsequently underwent oxidation to form interpeptide disulfide bonds (Figure 6C). The stabilized cross-linked peptide/pDNA condensates were found to be more potent at mediating gene expression.

Integration of bioreducibility and the buffering capacity were achieved by substituting His for Lys residues in Cys-Trp-(Lys)$_{17}$-Cys to yield Cys-His-(Lys)$_6$-His-Cys peptide which facilitated lysosomal trafficking and delivery of DNA to the cytosol resulting in successful gene expression in HepG2 cells (Figure 6D).[43]

For in vivo gene delivery cross-linked peptides were further functionalized with PEG and targeting ligands. Synthetic peptide possessing a single sulfhydryl group on the N-terminal cystein, and two or five internal acetamidomethyl (Acm)-protected cysteine residues, was reacted with either PEG vinyl sulfone or iodoacetamide tyrosinamide triantennary N-glycan. PEG-peptides, and glycopeptides were then conjugated via sulfhydryl cross-linking, and the subsequent spontaneous polymerization was achieved through disulfide bond formation during condensation with pDNA (Figure 6E).[44]

The Seymour group resorted to surface modification of complexes with multivalent coating with hydrophilic

copolymer of *N*-(2-hydroxypropyl) methacrylamide (PHPMA) with methylmethacryloylglycylglycine 4-nitrophenyl ester, which provided lateral stability into the peptide/pDNA complexes through the enhancement of the polyelectrolytic stability. The sophisticated approach to trigger the required dissociation of pDNA from complexes after cellular uptake, involved the development of reductively cleavable linear polycations (RPC), which was composed by oxidation of terminal cysteinyl thiol groups of $Cys(Lys)_{10}Cys$ peptides (Figure 6B). The laterally stabilized vectors which could undergo significant intracellular reductive degradation were able to compensate the lateral stabilization introduced by surface coating with PHPMA and thus facilitated facile release of the DNA.[45]

Functional peptides such as TAT, KALA, and NLS have also attracted considerable attention for their efficacies in mediating gene delivery. TAT-peptides, cell-penetrating peptides composed of 47−57 amino acid residues, possess an unusual ability to translocate across cell membranes in a receptor- and temperature-independent manner. Polyplexes based on polyTAT prepared by oxidative polycondensation, and TAT polymer prepared by in situ template-assisted polymerization, exhibited reduced cytotoxicity than PEI25K (Figure 6F).[46] HMW TAT polypeptides demonstrated increased transfection efficiency compared to control TAT peptide due to the increased charge density for condensing pDNA. The presence of TAT residues in polyplexes surfaces resulted in better interaction with cell membrane and subsequent cellular uptake led to enhanced gene expression.

Insufficient gene silencing ability of KALA, a fusogenic peptide, arising due to its instability in the presence of serum proteins was successfully addressed by imparting high cationic charge density to KALA peptides through installing self-cross-linking moieties such as disulfide linkages. The formation of stable and compact polyelectrolyte complexes of siRNA and siRNA-PEG conjugate with cross-linked KALA exhibited more favorable cell cytotoxicity profile and enhanced gene silencing efficiency (Figure 6F).[47]

Reducible copolypeptides (rCPP) fabricated by an oxidative polycondensation using an engineered histidine-rich peptide (HRP) and a nuclear localization sequence (NLS) derived from the SV40 virus endowed with endosomal buffering capability and nuclear localization capability, exhibited minimal cytotoxicity and transfection activity comparable to or better than PEI25K (Figure 6G).[48] The intracellular degradation of rCPP into short cationic fragments was thought to be responsible for the significant reduction in the cytotoxicity as the polycation toxicity depends on the MW.

Chemical Conjugation of siRNA with Disulfide Linkage

siRNA has been widely used as a gene silencing agent due to their sequence-specific mRNA cleavage ability, however vulnerability of siRNA toward enzymatic degradation warrants special attention. Various polymer−siRNA conjugates are reported to increase the serum stability of siRNA but are unable to address insufficient gene silencing, off-targeting, and immune responses. It is assumed that the steric hindrance offered by polymer to siRNA might prevent the formation of a RISC−siRNA complex leading to low gene silencing. Incorporation of disulfide linkages to the polymer−siRNA conjugate becomes advantageous as they can induce release of siRNA from polymer−siRNA conjugate in the intracellular region. siRNA linked to various nanocarriers through reducible linkages exhibited higher gene silencing efficacy than nonreducible siRNA systems.[49]

VEGF siRNA was conjugated to PEG via a disulfide linkage (siRNA-SS-PEG) to fabricate polyelectrolyte complex (PEC) micelles-based siRNA delivery system (Figure 6H).[50] The conjugation of siRNA to PEG via a disulfide bond was not only highly desirable from a steric standpoint of view, but the micellar formulation also greatly suppressed VEGF gene expression up to 96.5% in a highly sequence-specific manner. Such PEC micelles when administered through intravenous as well as intratumoral injection significantly inhibited VEGF expression at the tumor tissue and suppressed tumor growth in an animal tumor model without showing any detectable inflammatory responses in mice.[51]

To study the effect of cleavable disulfide linkage and the site of PEGylation in gene silencing, siRNA was conjugated with PEG at four different terminal ends (sense 3′, sense 5′, antisense 3′, and antisense 5′) via cleavable disulfide and noncleavable thioether linkages, and their gene silencing efficiencies were evaluated upon complexation with Lipofectamine2000.[52] Cleavable siRNA-SS-PEG conjugates showed comparable gene silencing activities to naked siRNA, and exhibited sequence-specific degradation of a target VEGF mRNA and inhibition of VEGF expression, whereas noncleavable siRNA-PEG conjugates exerted RNAi effect without any target sequence-specificity. Meanwhile the variation in PEGylation site at siRNA terminal ends did not affect gene silencing efficacy. Additionally, noncleavable siRNA-PEG conjugates induced nonspecific immune responses to slightly higher extent than cleavable siRNA-SS-PEG conjugates, underpinning the reductive cleavage of disulfide linkages in the cytoplasm as the probable factor

that decreased the chance of cytoplasmic immune activation. This result implied that reduction-mediated cleavable siRNA-SS-PEG conjugates would be utilized for efficient and safe siRNA applications.

As polymeric ionic complexes (PIC) of siRNA are not stable under the physiological condition, siRNAs are conjugated to polymer backbone to form stable PIC. Kataoka et al. fabricated siRNA-grafted poly(aspartic acid) [PAsp(-SS-siRNA)] to impart enhanced charge density and stability of siRNA on the polymer backbone and accomplished successful PIC-based siRNA delivery (Figure 6I).[53] PAsp(-SS-siRNA) facilitated the formation of stable PICs than those from monomeric siRNA due to the higher anionic charge density. Cleavage of the disulfide linkage took place under reductive conditions, thereby facilitated siRNA release from the PICs in the cytoplasm. PAsp(-SS-siRNA)/PAsp (DET) PICs induced strong gene silencing against luciferase without any significant cytotoxicity.

In an effort to overcome the general impediments in the nonviral siRNA delivery such as low charge density and rigid structure of siRNA, a new class of biologically active siRNA having chemically self-cross-linked[54] and multimerized siRNA was developed (Figure 6J).[55,56] Higher charge density and chain flexibility of multimeric siRNA conferred enhanced condensation ability, biocompatibility, and gene silencing when complexed with cationic polymers.

Conclusions

Bioreducible polymers provide a giant leap in the quest for an ideal gene delivery vector. The ever-continued endeavor in developing efficient delivery systems coupled with the acquired knowledge of molecular biology makes bioreducible polymers highly sought after tools in delivering therapeutic payloads especially the nucleic acids. However, the presence of several reducing agents such as GSH, protein disulfide isomerase (PDI), and phosphoglycerate kinase (PGK) in the extracellular environment,[11,57] even though in trace amounts, should be taken into consideration during the designing of disulfide-based gene carriers. Though there are several reports which demonstrated the mechanistic details pertaining to the kinetics and sites of disulfide reduction of small molecules in the subcellular compartments, there is a great need to study the subcellular location of disulfide-containing polymers.

In conclusion, practical realization of gene therapy in clinical practice warrants greater impetus in wider array of research domains encompassing bioreducibility, target specificity, serum stability, and intracellular trafficking. To this end, successful integration of such attributes is crucial to exploit the unique functional traits of bioreducible disulfide linkages. Therefore, future research activities are set to unearth the hidden possibilities of bioreducible nanocomposites in mediating efficient gene delivery.

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ABBREVIATIONS

PEI-SH; thiolated PEI; PEI-SS; disulfide cross-linked PEI; DSP; dithiobis(succinimidyl propionate); CBA; *N*,*N*′-cystamine bisacrylamide; PAA; poly(amido amine); SS-PAA; reducible poly(amido amine); SS-PAEI; reducible poly(amido ethylenimine); SS-PAED; reducible poly(amidoethylenediamine); DMC; *N*,*N*′-dimethylcystamine; EDC; ethylenediamine; PDA; poly(disulfide amine); poly(CBA-DAH); PDA containing hexaethylene spacer;

BIOGRAPHICAL INFORMATION

Sejin Son received her B.S. and M.S. in molecular science and technology at Ajou university in 2008. She is currently pursuing her Ph.D. research at the Department of Chemistry, Pohang University of Science and Technology (POSTECH) under the supervision of Prof. Won Jong Kim. Her main research interests concern the development of stimuli-sensitive drug/gene delivery systems covering various types of polymers and gold nanoparticles.

Ran Namgung received her B.S. in 2008 from the Department of Chemistry at Konkuk University. She is a graduate student pursuing her Ph.D. under the supervision of Prof. Won Jong Kim at the Department of Chemistry, POSTECH. Her thesis work involves the design and development of intelligent nanoconstructs utilizing polymers and nanoparticles for nucleic acid delivery and imaging.

Jihoon Kim received his B.S. in 2009 at the Department of Life Science, POSTECH. He is currently in the Ph.D. program working on designing and synthesizing various types of drug/gene delivery systems under the supervision of Prof. Won Jong Kim.

Kaushik Singha earned his doctoral degree (2004) under the supervision of Dr. S. B. Mandal, at Indian Institute of Chemical Biology. He joined Chembiotek Research International, India, as a Research Associate in 2004. Since 2008 he has been working with Prof. Won Jong Kim as a research professor in POSTECH, South Korea. Currently he is engaged in designing and synthesizing various nonviral polymeric vectors for efficient gene delivery.

Won Jong Kim received his B.S. from Hanyang University in 1998, and M.S. and Ph. D. in Biomolecular Engineering in 2004 at Tokyo Institute of Technology. During his graduate studies with

Profs T. Akaike and A. Maruyama, he developed a polymer-mediated DNA detection system. From 2004 to 2007, he was a postdoctoral fellow at the University of Utah under the supervision of Prof. Sung Wan Kim. Currently, he holds an associate professor position at the Department of Chemistry, Pohang University of Science and Technology (POSTECH).

FOOTNOTES

*To whom correspondence should be addressed. E-mail: wjkim@postech.ac.kr.

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