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KEYNOTE

# CRISPR/Cas9 in the era of nanomedicine and synthetic biology

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**The CRISPR/Cas system was first discovered as a defense mechanism in bacteria and is now used as a tool for precise gene-editing applications. Rapidly evolving, it is increasingly applied in therapeutics. However, concerns about safety, specificity, and delivery still limit its potential. In this context, we introduce the concept of nanogenetics and speculate how the rational engineering of the CRISPR/Cas machinery could advance the biomedical field. In nanogenetics, the advantages of traditional approaches of synthetic biology could be expanded by nanotechnology approaches, enabling the design of a new generation of intrinsically safe and specific genome-editing platforms.**

**Keywords: Genome editing; Nanomedicine; Synthetic biology; Nanogenetics**

## Introduction

The first two decades of the 21st century have seen spectacular advances in molecular biology. Of these, the discovery of CRISPR/Cas9 has contributed significantly to changing approaches to genome editing.[1] It has completely revolutionized our understanding of biomedical research, by allowing us to theoretically study, and even modify, the genome of almost any organism.[2,3] Indeed, the potential to treat genetic disorders directly could enable the development of novel therapies.[4,5] Despite such potential, there is still a long way to go. Although genome editing suffered setbacks during the late 1990s,[6] and is still associated with ethical concerns, it is merely a question of time until it is integrated as a therapeutic modality in the clinic.[7] This comes at a time when the CRISPR/Cas9 toolbox is continuously evolving to reduce associated constraints.[2,3] Depending on the gene-editing application and cellular target system, a suitable CRISPR/Cas technology has to be chosen. Both these parameters have to be considered during the final experimental design and are influenced by additional factors, such as the implemented delivery substrate and system, as well as the biological environment itself.[3] Our understanding of gene delivery systems, such as viral vectors, has grown considerably in recent years.[8] However, these natural shuttle systems, which are exploited to deliver the gene therapy machinery inside cells, are still associated with harmful drawbacks, such as immunogenicity, insertional oncogenesis, and off-target effects.[9] In this context, another emerging branch of science gaining increasing implementation is nanomedicine, which can be used to circumvent limitations of conventional methodologies. The specificity of nanomaterials lies in their chemical and physical properties, which are intrinsic to their composition, shape, and nano-scaled size.[10] Nanotechnologies are already implemented as medical diagnostics, monitoring

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tools, and treatment.[11,12] Indeed, more than 50 nanoformulations are on the market and more than 400 nanomedicine formulations are in clinical trials.[13] With regards to gene therapy, a lipid nanoformulation received regulatory approval after a 20-year wait for the first RNAi drug.[14] NTLA-2001, another emerging nanomedical therapeutic agent, was designed to treat transthyretin amyloidosis (ATTR) and was the focus of the first clinical study implementing the CRISPR/Cas9 technology *in vivo*.[15] These remarkable examples are among several that blaze the trail for a new generation of innovative gene therapy medications, which take advantage of the combination of the CRISPR/Cas9 system and nanotechnology.

However, the CRISPR/Cas9 system itself has intrinsic safety constraints, causing, for instance, unintended DNA modifications at nontarget genomic loci or cytotoxicity.[16] Thus, remote, controlled activation of the gene-editing machinery is important for many biological and medical applications. Given the complexity of most subcellular processes, full control is not always easily achievable. To overcome this general limitation, synthetic biology aims to develop tailor-engineered tools that can be exploited for specific purposes and be switched on/off.[17–19] For instance, several studies have focused on the rational engineering of the CRISPR/Cas machinery, and its canonical and noncanonical roles, favoring a range of diversified applications.[20–28] Within the next few years, approaches of synthetic biology harnessing the 'natural' benefits of the system, including its programmability, and combining them with modern technologies, are likely to become an important component in medical research. Thus, we envisage that the recent technological advances in the field could bring ground-breaking solutions in the near future. For instance, the design of tunable molecules and supramolecular complexes could switch gene-editing events on/off in a spatiotemporally precise manner by applying external stimuli. Gaining control over the activation or disengagement of the editing machinery could realistically improve the safety profile of the formulation, further amplifying its effective application spectra in therapeutics.

In this review, we briefly discuss the current opportunities and limitations of the CRISPR/Cas system. We highlight the advantages offered by a nanotechnology-based delivery and the rational design of CRISPR/Cas machinery in solving the open issues. We also introduce the concept of nanogenetics, an emerging discipline that combines nanomedicine and synthetic biology approaches for the design of programmable systems for gene editing, ensuring stable control at a high resolution.

## The CRISPR/Cas9 revolution

The CRISPR/Cas system was discovered in 2004 as a prokaryotic adaptive immune mechanism that protects archaea and bacteria from bacteriophage and mobile genetic element infections,[29–32] targeting and cutting foreign genomes with the RNA-guided endonuclease Cas.[33] Soon after, the CRISPR/Cas system was re-engineered for biotechnology applications. Given the unique simplicity and flexibility of the CRISPR/Cas technology, this system has been used for diverse applications (Fig. 1, inner circle). Among the different Cas proteins, *Streptococcus pyogenes* Cas9 (SpCas9) is the most widely used because of its simple application design. In combination with a specific synthetic guide, the sgRNA, the system is an easily achievable and efficient gene-editing tool.[1] For instance, genetic engineering requires the design of the 20-nucleotide sequence in the sgRNA, resulting in sgRNA–DNA pairing when the protospacer adjacent sequence (PAM) is present on the nontarget DNA strand, proximal to the cleavage site.[34] Upon target recognition, the Cas9 HNH (Histidine-Asparagine-Histidine, His-Asn-His) nuclease domain cuts the target strand whereas the RuvC-like nuclease domain cleaves the nontarget strand, generating a double-strand break (DSB).[1,33,35]

The crystal structure of SpCas9, associated with sgRNA and target DNA, has revealed detailed information on the mechanism of complex formation and function.[36–38] The Cas9 architecture comprises two lobes: the nuclease lobe, containing the RuvC-like and the HNH domains, as well as the C-terminal PAM recognition domain, and the α-helical recognition (REC) domain, which is essential for binding to the sgRNA and the target DNA.[36–38] The binding of the sgRNA to Cas9 induces conformational changes that reorient the two lobes, generating a channel to accommodate the target DNA and, thus, representing a stringent prerequisite for its activation.[36,37]

Generation of the DSB activates the DNA damage response and cellular mechanisms for DNA repair. In eukaryotes, DSBs are mainly repaired by the error-prone nonhomologous end joining (NHEJ) mechanism, because the homology-directed repair (HDR) system is restricted to the G2 and S phases of the cell cycle.[39] Whereas the exploitation of NHEJ for gene editing determines the incorporation of small insertions or deletions (indels, producing frame-shifts or premature stop codons) and even translocations, HDR can be used to introduce a specific modification by providing a donor DNA template containing the modified sequence (point mutations or insertions) flanked by sequences homologous to the upstream and downstream target region (Fig. 1a). Activation of NHEJ following Cas9 DNA cleavage has been used to create gene knockouts and perform high-throughput screens to study gene function *in vitro* and *in vivo* in animal models, while gene editing activating the HDR pathway has allowed for the correction of mutations in genetic diseases (reviewed in [40,41]).

### *Next-generation gene editing*

Alternative strategies that do not involve the generation of DSBs have been developed exploiting, for instance, the partial or complete inactivation of Cas9 nuclease activity. In particular, mutations in one of the two Cas9 nuclease domains (either mutation of the aspartic acid 10 to alanine, D10A, in the RuvC-like domain or of histidine 180 to alanine, H840A, in the HNH domain) result in Cas9 nickases [nCas9 (D10A) and nCas9 (H840A)], which are able to generate single-stranded DNA nicks.[1] At the same time, introducing both mutations completely abolishes the Cas9 nuclease activity while retaining its binding capacity, characterizing the 'dead Cas9′ (dCas).[42] Both dCas9 and nCas9 have been used in the development of base editors to introduce site-specific editing without producing DSBs, reducing the cytotoxic effects of Cas9 nuclease (reviewed in [43]). Base editors direct the conversion of single nucleotides using nCas9 or dCas9 fused with deaminases to convert a cytosine-guanine base

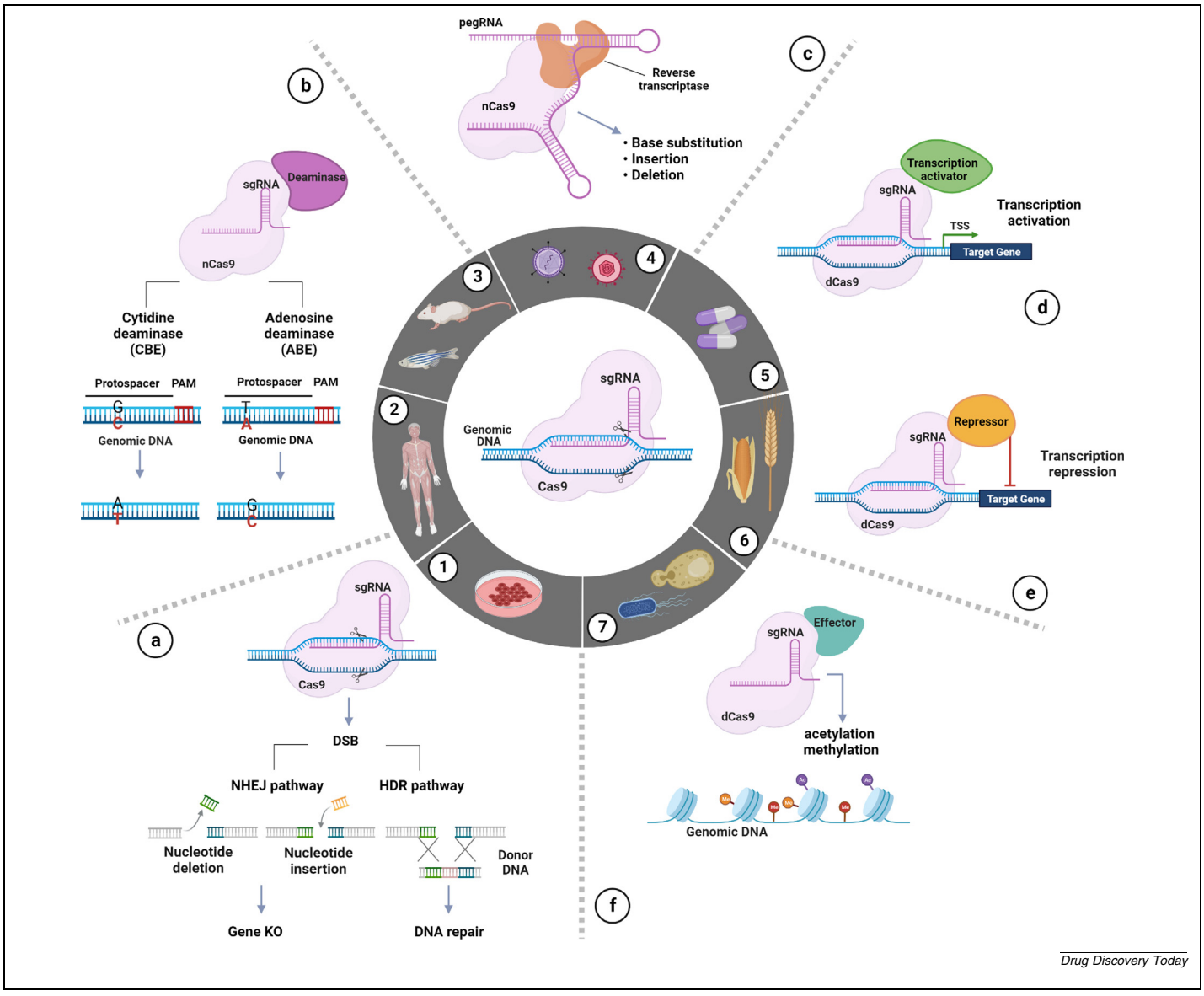



**FIGURE 1**

Applications of CRISPR/Cas9 technology. The inner circle shows the different fields of application: (1) basic research, (2) human biomedical research and therapies development, (3) animal model production, (4) viral infection treatments, (5) drug targets and resistance identification, (6) plant biotechnology, and (7) microbial cell factories for industrial biology. The outer circle summarizes the CRISPR/Cas9-editing strategies: (a) gene editing, exploiting either the nonhomologous end joining (NHEJ) or homology-directed repair (HDR) repair pathway, (b) CBE and ABE base editors, (c) prime editors, (d) CRISPRa and (e) CRISPRi for transcription regulation, and (f) epigenetic editing for chromatin modification.

pair (C•G) into thymidine-adenine base pair (T•A) (cytidine base editors, CBEs),[44] or adenine–thymidine base pair (A•T) transition to guanine–cytosine base pair (G•C) (adenine base editors, ABEs)[45] (Fig. 1b). CBEs and ABEs cover all four base transitions (C to T, A to G, T to C, and G to A); however, base editors cannot perform all 12 different base modifications, restricting their potential for certain substitutions. Nevertheless, they are particularly promising to correct genetic diseases caused by single-base substitutions.[46,47]

Prime editing (PE) instead allows for targeted small deletions or insertions, and all transition and transversion modifications without introducing DSBs or requiring donor DNA (Fig. 1c).[48] The PE system relies on the target specificity of the Cas9:sgRNA and the nickase activity of Cas9; in addition, the system requires a modified sgRNA guide (pegRNA) comprising an extension edit-containing template, and the Cas9 (H840A) nickase C terminus fused to the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT). The Cas9 nickase cuts the DNA exposing a 3′-hydroxyl group, which can be used to prime the RT using the primer binding site and the edit sequence on the pegRNA.[48]

Beyond gene editing, the CRISPR/Cas9 system has been exploited to study and reprogram gene regulation. The catalytically dead Cas9, (dCas9),[42] which retains DNA-binding ability, allowed for the development of new tools to control gene expression, interfering with transcription or epigenetic modifications.[23,49] Fusion of the dCas9 to transcriptional activator[50] or

repressor domains[51] resulting the CRISPR activation (CRISPRa) (Fig. 1d) and CRISPR interference (CRISPRi) (Fig. 1e) tools,[23,52] confered the ability to alter gene regulation without permanent modification of the genome. In addition, Cas9 fused to the catalytic domain of chromatin modifiers has been exploited for epigenetic editing[23,53,54] (Fig. 1f), allowing the pattern of epigenetic modifications of DNA and histones to be altered on specific promoter or enhancer regions. Another approach to modulating gene expression that does not involve DSBs (and NHEJ pathway activation) is the use of a type VI Cas nuclease, Cas13. This precise and programmable nuclease targets and cuts RNA once assembled with a specific sgRNA molecule. Consequently, it displays a high level of target specificity to knock down gene expression without inducing permanent DNA alterations.[55]

## The CRISPR/Cas9 system in medicine: Limitations

Despite the simple and flexible design of the CRISPR/Cas system, there are important limitations to the use of this technology in medical applications. Mutations at sites other than the intended one (i.e., off-target events) are a major concern. Mismatches of one to five base pairs in the sgRNA can be tolerated by Cas proteins, causing unwanted cuts.[56,57] The accurate design of the sgRNA is a determinant for reducing off-target events and, therefore, has been a focus of intense research and the development of software aiming to predict and design the appropriate sgRNA.[16] Here, fundamental characteristics are an enhanced DNA-target binding efficiency (maximized ON-target) coupled with a high target specificity (minimized OFF-target). Both properties are directly influenced by the structure of the target site itself, requiring further precise analysis of its features to reduce unintended cleavage or the introduction of mutations at similar, but not identical, genomic sites.[58] Off-target sites are often not reported by prediction software, because they contain either four or more single nucleotide or insertion/deletion mismatches.[59] Cas9 variants with reduced off-target activity have also been developed by reducing the affinity of Cas9 for DNA while maintaining or enhancing the role of the DNA:RNA heteroduplex in the editing process.[16]

The requirement for PAM near the cleavage site restricts the number of potential target sequences on genomes. Advancements have been made to control and reduce this limitation, such as the use of different Cas9 orthologs that recognize different PAM sequences (reviewed in [41,60]) or the engineering of Cas9 variants able to recognize a broader range of PAM motifs.[16,61]

For the CRISPR/Cas9 system to be used in clinical applications, the delivery of the Cas9:sgRNA complex *in vivo*[62] has to fulfil two major requirements: (i) to deliver the CRISPR machinery with high efficiency to the target site while restricting its intracellular entry to the targeted cells; and (ii) to elicit a low immunogenic response and minimal toxic effects. In addition, the delivery strategy has to take into account encountered barriers, as well as the entry route of the cargo into the cell and its intracellular trafficking (i.e., endosomes, lysosomes) to the nucleus.

Currently, most research efforts are focused on: (i) using synthetic biology-based engineering strategies to strictly constrain the Cas9:sgRNA complex activity and control its expression and localization in time and space; and (ii) using nanomedicine-based approaches for developing effective approaches for an efficient and targeted *in vivo* Cas9:sgRNA delivery causing minimal immunogenicity and toxicity.

Table 1 summarizes current limitations. Nanomedicine and synthetic biology can provide distinct solutions for addressing specific issues, as discussed below. We also speculate about how nanogenetics can provide an integrated approach for the design of a 'fix-all solution'.

## Impact of nanomedicine on CRISPR/Cas9 gene editing

### *Delivery issues in therapeutic applications*

Delivery of the CRISPR/Cas machinery could occur in the form of nucleic acids (plasmids or mRNA) or the RNP complex itself (Fig. 2a).[63] Although the delivery of a plasmid encoding the Cas9 and sgRNA allows generally for robust and stable expression, administration of the CRISPR/Cas9 machinery in the form of RNP offers major advantages, especially in terms of safety, a key determinant for medical applications. Indeed, once inside the cell, the already assembled RNP complex allows for a rapid onset of action, entailing high genome-editing efficiency; importantly, the complex has a reduced activity period of a few hours because of its half-life, allowing for rapid clearance.[64] By contrast, when administered in the form of nucleic acids, the complex will persist for longer because it will be expressed in the target cells.

**TABLE 1**

**Potential approaches to overcome CRISPR/Cas-associated drawbacks to expand its clinical application.**

| Current limitation | Possible solution from: | |
|---|---|---|
| | Nanomedicine | Synthetic biology |
| Poor *in vivo* delivery | Design of drug delivery systems | |
| Nonspecificity of treatment | Strategies for targeted delivery | |
| Low fidelity of nuclease | | Selection of high-fidelity variants |
| Immunogenicity of viral vectors | Use of nonviral vectors | |
| CRISPR/Cas administered in form of plasmids: toxicity from Cas constitutive expression | | Transient expression of Cas proteins |
| CRISPR/Cas administered in form of RNP: poor stability and penetration | Nanoformulated RNPs show increased stability and penetration | |
| Endosomal degradation of complex | Strategies for endosomal escape | |
| Off-targets events | | Controlled activation/disengagement of editing |
| Limited PAM site | | Cas protein re-engineering |

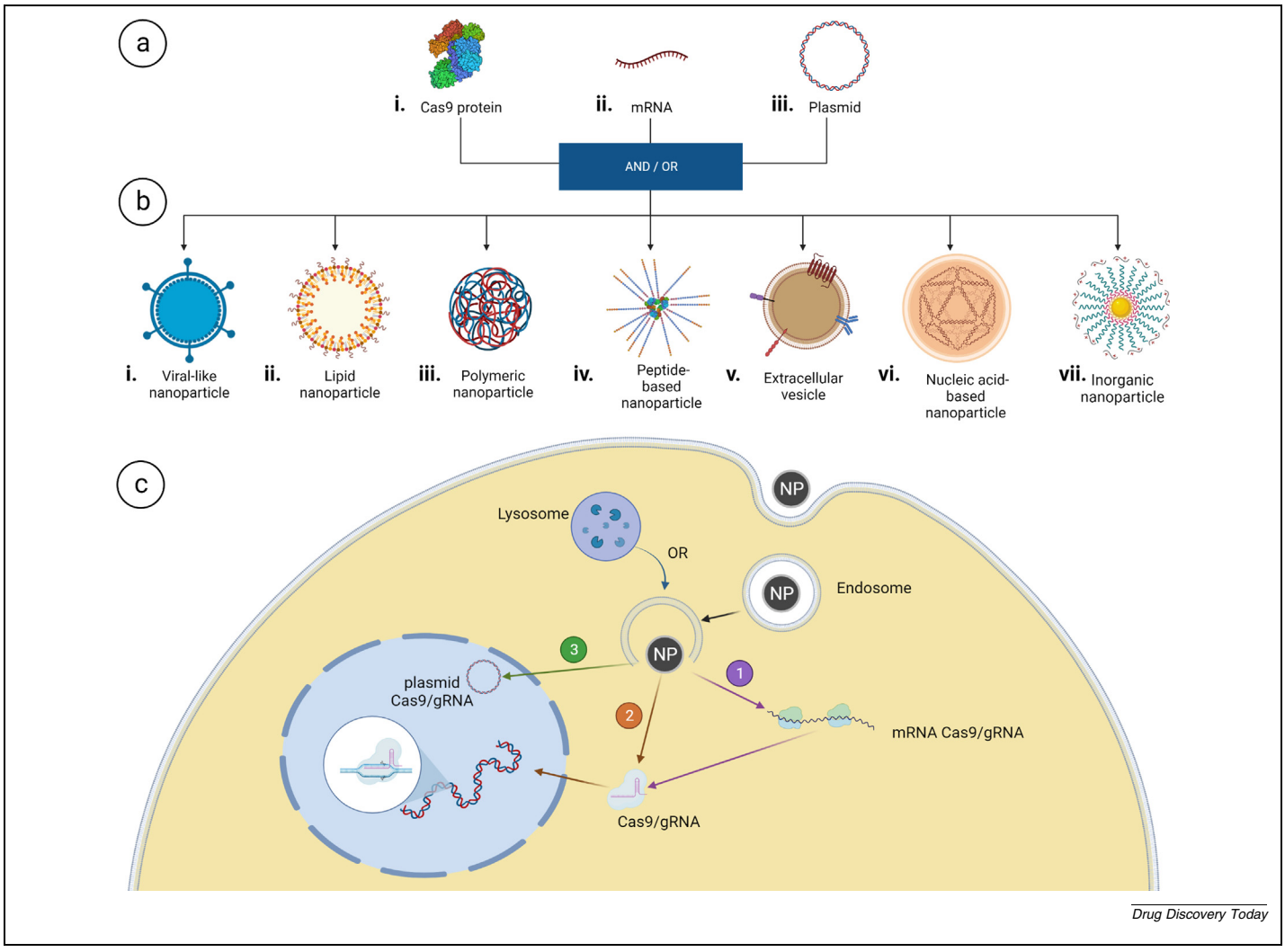



**FIGURE 2**

Nanotechnology-based delivery methods for the CRISPR/Cas9 complex. (a) Different forms under which the CRISPR complex is commonly associated with nanoparticles. (i) The Cas9 protein, (ii) mRNA, and (iii) plasmid DNA encoding the Cas enzyme and/or its guide (g) RNA. (b) Representative schemes of distinct nanoparticle (NP) classes (i–vii) for *in vivo* delivery of the CRISPR/Cas9 system. (c) Illustration of the cellular uptake and release of the cargo inside cells. The assembly of the Cas9/gRNA complex and its eventual transportation to the nucleus are shown. (1) The mRNA encoding the Cas9/gRNA is released into the cytoplasm and translated to form the ribonucleoprotein (RNP) complex, which is then translocated to the nucleus. (2) The already-assembled RNP complex delivered by the NP travels directly to the nucleus for gene editing. (3) Internalized plasmid DNA is transported to the nucleus, where it is further processed.

Here, the increased longevity of the gene-editing machinery is directly associated with a greater incidence of off-target activity, elevated immunogenicity, and cytotoxicity.[63] At the same time, successful gene therapy requires an appropriate concentration of the gene-editing complex. Thus, the implemented format of the cargo and its vehicle determine strongly both the gene-editing efficiency and the establishment of potentially harmful side effects.

To date, different strategies have been developed and explored for *in vivo* and *ex vivo* delivery, all of which have advantages and drawbacks. In general, they are mainly divided into three groups: physical, viral, and nonviral.[63,65,66]

Among the physical methods, the one most commonly used for the administration of the CRISPR/Cas9 machinery in *ex vivo* approaches is electroporation.[67] This technique involves the isolation of patient cells, their editing, and their transfusion to the patient from whom they originated (autologous transplant), or to a different patient (heterologous transplant). For instance, this strategy has been adopted in the treatment of two monogenic diseases: severe sickle cell disease (SCD) and transfusion-dependent β-thalassemia (TDT).[68] In both cases, patients receive autologous transplants of hematopoietic and progenitor stem cells edited by CRISPR/Cas9, and both approaches are in clinical trials (NCT03655678, NCT03745287). The heterologous transplant of donor CRISPR/Cas9-modified T cells can be used in chimeric antigen receptor (CAR)-T cell immunotherapy.[69] Although *ex vivo* approaches yield important benefits because they do not directly expose the patient to the gene-editing tool and allow for external quality control of the edited cells before their reintroduction, their application is limited to specific cell types and a limited number of diseases.[16]

Thus, to broaden the application spectra of the CRISPR/Cas toolbox, the big challenge and major goal in the field remains the development of efficient delivery strategies for *in vivo* gene

editing. Viral vectors have been widely exploited for delivery and gene therapy approaches (Fig. 2bi).[65,66] One of the most used viral strategies for the delivery of CRISPR/Cas9 is based on the use of adeno-associated viruses (AAVs). A remarkable example of this application is EDIT-101, an AAV5-CRISPR/Cas9-directed therapy, administered by subretinal injection for the treatment of Leber's Congenital Amaurosis Type 10 (NCT03872479). Although AAVs represent an excellent platform for gene therapy in light of their high transduction efficiency and low immunogenicity, they have important limitations. In particular, the limited packaging capacity of the AVV, which is ~4.7 Kb, poses a challenge because the SpCas9 together with its sgRNA is ~4.2 Kb in size. Other important issues include a detrimental increase in potential off-targets because of insertional mutagenesis, and long-term Cas9 expression in the transduced cells.[70,71]

### *Nanotechnology for in vivo delivery of CRISPR/Cas9*

Nanotechnology could help to address some of the crucial drawbacks of traditional delivery systems. The stabilizing and solubilizing effects they have on their cargo, paired with a high internalization rate, may indicate why nanoparticles (NPs) are considered the ideal carrier systems.[71,72]

Recently, NP-based carriers of the CRISPR/Cas9 machinery have been extensively implemented for *in vivo* and *ex vivo* applications.[71]

Among the different NP-based strategies developed and exploited so far, the most advanced are based on lipid NPs (LNPs) (Fig. 2bii). LNPs comprise amphiphilic molecules composed of hydrophilic headgroups and hydrophobic tails that can self-assemble in different structures in aqueous environments depending on the geometry of the amphiphile.[73] Cell targeting and internalization efficiency can be enhanced by mixing cationic lipids with cation surfactants, phospholipid emulsifiers, polyethylene glycol (PEG), fatty acids, and cationic peptides in liposomal formulations.[74] For example, cationic LNPs have been exploited to encapsulate the genetic information of CRISPR/Cas9 or the RNP complex itself and to deliver it to specific target cells.[75–77] In addition, liposomal carriers entrapping the genetic information of Cas9:sgRNA have been shown to display a prolonged therapeutic activity concomitant with a broad *in vivo* biodistribution range.[78] Moreover, modification of the surface charge, structure, and position of the ester in the lipid tails, and optimization of the structure of the head group demonstrated high levels of expression of the mRNA cargo, rapid tissue clearance, and high tolerability in mouse, rat, and nonhuman primates.[79] Implementing, for instance, the hydrophobic tails of liposomal molecules with reducible disulfide bonds is correlated with endosomal escape.[71] Indeed, LNPs engineered to display a high fusogenic behavior, demonstrated increased endosomal escape and cytoplasmic translocation of their nucleic acid payload and, therefore, higher efficacy.[80] One of the most exciting applications of LNPs has been the liver-targeted *in vivo* delivery of therapeutic RNA cargoes, such as small interfering RNA (siRNA) and mRNA. Here, a rational approach was used to design cationic lipids with a high affinity for hepatocytes by a specific opsonization process.[81] Specifically, absorption of apolipoprotein E (ApoE) on the surface of the LNPs facilitated lipoprotein receptor-mediated endocytosis into hepatocytes.[82] This research paved the way for the clinical development of patisiran, an RNAi-based therapeutic agent with the ability to improve the clinical symptoms of patients with hereditary transthyretin (TTR) amyloidosis.[83] The new frontier of this outstanding research is represented by NTLA-2001, an *in vivo* gene-editing therapeutic agent designed to perform a durable knock out at the level of the genomic locus responsible for the establishment of hereditary TTR amyloidosis. Strikingly, the formulation was efficient in patients even after a single injection.[15]

NPs can be conjugated with specific biomarkers to enhance their targetability. For example, polymer-based NPs (PNPs) (Fig. 2biii) exploit electrostatic interactions between cationic polymers and the Cas9:sgRNA complex[74] and can be easily modified by the incorporation of ligands, aptamers, or antibodies to interact with specific cell receptors.[84] Importantly, several members of this class appear to achieve endosomal escape by applying a proton sponge mechanism that culminates in the rupture of the organelle.[84] A practical example is given by a recent study focusing on the development of a novel gene therapy approach based on PEI-coated polymeric NPs. By modifying the PEI with β-cyclodextrin (β-CD), the researchers achieved enhanced delivery concomitant with reduced cytotoxicity, enabling the usage of this system in the treatment of β-thalassemia.[74,85] DNA nanoclews (DNA NCs) coated with PEI have been proven to be efficient in endosomal escape and were used successfully as a delivery vehicle for Cas9-sgRNA.[86] Moreover, Cas9-sgRNA can form nanocomplexes with different peptides, such as cell-penetrating peptides (CPPs), to improve cell penetration (Fig. 2biv).[87,88] For instance, the synthetic CPP, PepFect14 (PF14), enables the delivery of large fusion proteins while displaying a high transfection and editing rate.[89,90] Furthermore, CPPs allow for a more predictable cargo release, because they are responsive to the pH shift in the tumoral microenvironment.[91]

Scientists are increasingly exploring nature-inspired approaches for cell targeting that appear to be highly promising. Extracellular vesicles (EVs) (Fig. 2bv) are NPs of natural origin that can present an innate tropism for the cell population they originated from. EVs can be exosomes arising from the endosomal pathway, or microvesicles from the plasma membrane and can encapsulate several types of biomolecule.[71] For example, cancer-derived exosomes or microvesicles are already being exploited for the delivery of the Cas9 protein and sgRNA. Given their maternal cell tropism, EVs target cancer cells efficiently, into which they deliver their cargo, thus are a promising tool for anti-cancer therapy.[92,93] To increase further their target specificity, EVs can be surface modified with ligands or peptides,[94] or specific DNA aptamers.[95]

### *Nanomedicine research is moving quickly toward stimuli-responsive CRISPR/Cas9 delivery*

Recently, an LNP-based strategy combining cell-targeting specificity with stimuli-responsiveness has emerged that allows for the controllable release of the payload. For instance, pH-sensitive cationic liposomes designed with Cas9-gRNA-HPV 16 E6/E7, and targeting cervical cancer, have been shown to inhibit tumor growth in mice, while displaying further reduced lysosomal degradation, immunogenicity, and toxicity.[96] In this regard,

novel nanoformulations with similar properties are under investigation. Smart polymeric systems with the ability to be stimuli regulated are gaining increasing attention. This kind of NP usually responds to a variety of cues either by swelling or collapsing, enabling multifunctional applications.[84] PNPs responsive to pH shifts, the redox environment, reactive oxygen species (ROS), temperature, enzyme activities, osmotic phenomena, or even light, are already in use.[97]

DNA origami (Fig. 2bvi) has also been proposed as an alternative stimuli-responsive strategy. DNA origami comprises DNA strands that can self-assemble in 3D cages through complementary sequence design.[98] They can be designed to unlock their cage in response to a specific interaction between an aptamer sequence and a miRNA. For instance, miRNA-responsive DNA origami has been used for the delivery of the RNP complex. The RNP can be efficiently loaded by sequence hybridization between the DNA and the gRNA. The base pairing can be then replaced, for instance, by a tumor-specific miRNA, allowing the release of the RNP from the DNA cage in the target tumor cells.[99]

Another interesting category of stimuli-responsive materials is that constituted by inorganic NPs (Fig. 2bvii). For instance, gold nanoparticles (AuNPs) have been extensively exploited for the delivery of the Cas9 protein and sgRNA, showing high internalization efficiency, even in difficult-to-transfect-cell-lines, both *ex* and *in vivo*.[100] Moreover, AuNP assembly and disassembly can be triggered by pH,[101] or their cargo release can be photo-triggered under laser irradiation.[102] Furthermore, advances in nanotechnology have resulted in another interesting class of clinically feasible NPs, termed ultrasonic NPs (UNPs), which have been successfully used to deliver genetic materials with high fidelity.[103] In addition, because UNPs are responsive to acoustic waves, they can be exploited to increase membrane permeabilization, promoting localized cellular uptake.

## Approaches of synthetic biology to genome editing

### *Why synthetic biology*

The increasing use of CRISPR/Cas technologies in genome editing and gene expression regulation requires ways to restrict and control the activity of this potent tool. More specifically, this technology must be strictly confined especially in its dose, and in its temporal and spatial dimensions, before being suitable for translational medicine applications. In this context, repurposing the CRISPR/Cas system could not only alleviate potentially harmful side-effects, for instance by reducing off-targets and genotoxicity, but also increase its selectivity and therapeutic efficacy.[104]

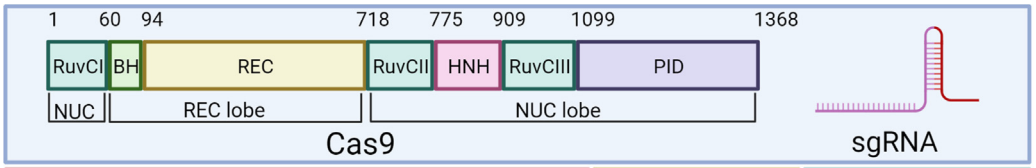
Synthetic biology is characterized by combining the classical biological approach of top-down research with an opposite, engineering perspective, defined as bottom-up. It focuses on two major aspects: the development of novel, non-natural entities, and the redesign of existing ones aiming to unravel or modulate the mechanisms that govern biological systems.[17] Consequently, it offers completely new opportunities, because the recent advantages of *in vitro* and bioengineering techniques allow for the design of tailored versions of nature-derived building blocks. These fine-tuned biological entities can be applied for a plethora of useful applications, from industry to research, biotechnology, and medicine.[105] Moreover, synthetic biology enables the limitations intrinsic to biological systems to be overcome because it increases their potential by providing novel functions.[17] Given that genetic engineering is defined as the deliberate modification of the genetic information of an organism, it is hardly surprising that it forms part of the synthetic biology toolbox.[28] This classification becomes even more evident with regards to the emerging field of CRISPR/Cas9 technologies, which aim to not only edit, but also reprogram the genetic make-up, creating entities that could thus deviate from their native conformation.[28]

As discussed above, CRISPR/Cas9 has become an important system, mainly because of its simple two-component design, the sgRNA and the Cas nuclease, resulting in the increased evolution of this powerful gene-editing tool. Consequently, efforts have been made to optimize these two components in particular to expand their activity range and augment specificity and safety (i.e., by the creation of a customized chimeric sgRNA molecule or selection of new Cas variants) (Fig. 3a–c). However, this topic is addressed elsewhere [41,61,106] and is not further discussed here. Instead, we focus our attention on the strategies for switching on and off the system activity in a controllable fashion.

### *Protein engineering for controlled activation*

Harnessing biological systems to construct predictable and controllable ones is the basis of synthetic biology research. Distinct research groups focused their attention on the development of an endogenous transcription system, comprising a dCas9, whose activity is restricted by the presence of light-responsive transactivator domains, and an effector protein.[24,107] These studies used plant-derived blue light-responsive heterodimerization domains (CIB1 and CRY2 from *Arabidopsis thaliana*) but differed in the choice of the transcriptional activator used. Both parts of the system were equipped with a distinct partner domain, which dimerized upon stimulation, enabling synergistically enhanced gene expression in a temporospatially defined manner in living cells (Fig. 3d).[24,107] These approaches are important examples showing how modular genetic engineering enables easily achievable reversible control of photo-induced effector proteins to regulate intracellular mechanisms. Another prominent trigger exploited in non-invasive and reversible stimulation of synthetic biology constructs is temperature. The design of a gene switch responsive to a temperature shift integrated into the dCas9 system enables, for example, the remote modulation of transcription.[108] While engineered dCas9 constructs have been mainly exploited in the study of gene expression, the controlled targeting of effector proteins to desired genomic locations was investigated by Chen and colleagues, who focused on temporally confining epigenomic editing.[109] They exploited a chemically responsive system together with a synthetic dCas9 enabling precise temporal and gene locus-specific editing (Fig. 3e). The authors relied on a naturally occurring signaling molecule, abscisic acid, to control the functional interaction of the distinct components of the system.[109] Recently, other chemically inducible constructs have been developed that allowed not only for accurate evaluation of the kinetic behavior of the construct itself in a temporally precise manner, but also to measure the editing efficiency.[110]

With the significant progress witnessed over the past decades in genomic decoding and proteomics, split proteins have gained




| RE-ENGINEERING | WHAT | WHY |
|---|---|---|
| a | PAM interacting domain | Recognize a broader range of PAM motifs [61,106] |
| b | Chimeric sgRNA molecule | Targeting to a defined DNA sequence [106] |
| c | Catalytically dead Cas9 (dCas9) | Interference [23] |
| d | Light-responsive heterodimerization domains CIB1 and CRY2 fused with dCas9 and an AD, respectively | Light-responsive transcriptional activator [24,107] |
| e | Abscisic acid (ABA)-inducible binding between PYL and ABI proteins, fused to dCas9 and P300 HAT, respectively | Chemically-responsive epigenomic editing [109] |
| f | Split Cas9 reassembles into a completely functional unit upon interaction with its native sgRNA | sgRNA-inducible gene editing [111] |
| g | Split Cas9 reassembles upon dimerization of the two blue light responsive MAGNET domains | Light-responsive gene editing [112] |
| h | Split Cas9 reassembles only upon reconstitution of the two rapamycin responsive domains (FRB and FKBP) | Drug-responsive gene editing or gene expression redulation [113] |
| i | FRL-dependent expression of the nCas9-Coh2, costitutive expression of cCas9-DocS | Far-red light (FRL)-activatable gene editing [114] |
| j | Acr (anti-CRISPR proteins) inhibition of Cas9 and optogenetic release of the block | Blue light-activatable gene editing [20,120, 121] |

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increasing use in the life sciences. Here, the native protein is separated into two nonfunctional fragments, the heterodimerization of which is induced only under specific conditions, enabling regulable functional recovery. This basic concept of conditional reconstitution results in a powerful instrument for controlling and manipulating biological systems. In 2015, Wright and colleagues designed an inducible Split Cas9, which reassembles into a completely functional unit only upon interaction with its native sgRNA (Fig. 3f).[111] However, because split proteins also show a certain propensity to reconstitute in the absence of the triggering factor, the need for an additional layer of security became clear. Consequently, several studies were performed focusing on the integration of a split Cas9 with engineered nature-inspired fusion domains allowing for correct reassembly only in response to a specific stimulus, which could be of a natural or chemical entity. For example, Nihongaki and colleagues used two proteins of fungal origin in the design of a photo-activatable Split Cas9 to confine its spatiotemporal window of activity (Fig. 3g).[112] These two domains, named Magnets, bind to each other through electrostatic interactions, resulting in a non-invasive and reversible approach, because they are responsive to blue-light irradiation, which is of particular importance especially for *in vivo* applications. Zetsche and colleagues developed a chemical stimuli-responsive Split Cas9 for mediating gene editing and transcription.[113] Moreover, they used two domains, the FK506-binding protein 12 (FKBP) and the FKBP rapamycin-binding domain (FRB) of the mammalian target of rapamycin (mTOR), which were fused to the C- or N-terminal portion of the Cas9 protein, respectively (Fig. 3h). To further reduce the rate of spontaneous auto-assembly of the constructs, they equipped the N-terminal portion of one of the split Cas9 proteins with a single nuclear export sequence (NES) to sequester it spatially. Upon treatment with rapamycin, the two responsive domains (FRB and FKBP) fused to each Split Cas9 heterodimerize, and the complete entity is translocated in the nucleus because of the presence of the predominant nuclear localization signal (NLS) localized onto the C-terminal portion of the enzyme. By applying the same approach to a dCas9, the authors expanded the application of this tool to gene expression regulation.[113] These studies illustrate how researchers are trying to exceed the limits of natural evolution for useful purposes by relying on synthetic biology. Indeed, a few years later, Yu and colleagues developed a far-red light (FRL)-activatable Split Cas9, which carries the advantage of being able to penetrate deeply into tissue while displaying reduced phototoxicity; thus, it is better suited for clinical research and translational medicine applications than previous versions (Fig. 3i).[114] The authors further extended the technology by designing a split version, in which only one fragment is constitutively expressed, while the other half is regulated by a chimeric promoter, which itself is under the control of FRL-inducible synthetic transcription factors.[114] This modular scheme enables highly precise spatiotemporal control of gene editing, while reducing extensively the potential side effects on multiple scales.

### *Protein engineering for controlled disengagement*

While research is often focused on finding ways to activate a Cas9 system, the opposite approach, aiming to completely remove the complex, is less explored. Designing a Cas9 that would be inactivated once it has fulfilled its duty would further reduce the off-target score. Fusing Cas9 to small domains responsible for targeting the complex to intracellular degradation pathways in response to external stimuli could be advantageous, especially in cases where the activity of the system should be temporally limited to certain cellular populations.[115] Senturk and colleagues exploited a ligand-responsive destabilization domain that enables conditional and reversible gene editing at distance. In a dose-dependent manner, the cell-permeable ligand Shield-1 prevents the complex, which has been equipped with a synthetic FKB12 substrate, from proteasomal degradation, thus enabling its intracellular accumulation and endonuclease activity.[116] A similar but opposite approach considers the design of synthetic sgRNAs, which are fragmented upon irradiation, rendering the system nonfunctional after receiving the stimulus.[117] Indeed, precise genome editing does not only require high levels of specificity, but also safeguards.

A different option for inhibiting the activity of CRISPR/Cas is offered by nature. Anti-CRISPR proteins (Acr), which were identified in bacteriophages, are naturally occurring counteractors to bacterial Cas effectors.[118,119] Relying on diverse molecular mechanisms, these small proteins usually act by sequestering the CRISPR/Cas machinery, disabling its activity.[119] However,

**FIGURE 3**

The synthetic biology toolbox. SpCas9 domains are shown: α-helical recognition (REC) domain, bridge helix (BH), protospacer adjacent sequence (PAM)-interacting domain (PID), HNH, and RuvC. (a) A broader range of PAM motifs can be recognized by mutagenizing the PID.[61,106] (b) The creation of a customized chimeric single guide (sg)RNA molecule targets the ribonucleoprotein (RNP) to a defined DNA sequence.[106] (c) The dCas9 has a mutation in the HNH domain, which impairs the nuclease activity but not the helicase activity responsible for binding to the target region: targeting a DNA sequence (e.g., a promoter) could physically impede the transcription (interference).[23] (d) A light-responsive transcriptional activator can be generated by fusing light-responsive heterodimerization domains (e.g., CIB1 and CRY2) to the dCas9 and an activation domain (AD), respectively.[24,107] (e) The abscisic acid-responsive binding between the CYL and ABA proteins can be used to generate chemically responsive epigenomic editing by fusing CYL to the dCas9 and ABA to a histone acetyltransferase (HAT) (e.g., P300).[109] (f) Split Cas9 concept: the N-terminal and the C-terminal reassemble into a completely functional unit only upon interaction with its native sgRNA.[111] (g) Split Cas9 reassembles into a completely functional unit only upon blue light-inducible dimerization of the nMAG and pMAG domains. (h) Split Cas9 reassembles into a completely functional unit only upon rapamycin-induced dimer formation of FK506-binding protein 12 (FKBP) and the FKBP rapamycin-binding domain (FRB) protein domains.[113] (i) Split Cas9 reassembles into a completely functional unit only upon dimer formation between Coh2 (fused to the N-terminal fragment) and DocS (fused to the C-terminal fragment). The expression of nCas9-Coh2 is constitutive, while the expression of DocS-cCas9 is light (far-red) inducible.[114] (j) Anti-CRISPR proteins (Acr) can block the catalytic activity of Cas9 by binding to the HNH domain. Blue light-inducible release of the inhibition can be generated by fusing Acr to the LOV2 domain.[20,120,121] Schemes are illustrative and may not reflect the exact coding sequences used in the original paper.

their application spectrum is limited to inherent variants, thus reducing their implementation.[120] A recent study resolved this problem by taking advantage of synthetic biology. By designing synthetic Acrs, researchers were not only able to improve their functional capacity, but also to further expand their usage range to various Cas effectors.[120] They focused, for instance, on AcrIIC1, a relatively weak inhibitor that blocks the catalytic activity of Cas9 by binding to the highly conserved HNH domain. Next, by using standard synthetic biology techniques, the authors generated a chimeric protein with an increased targeted inhibitory effect.[120] Designing and developing biologically functional synthetic components that exceed their natural counterparts in terms of effectiveness is one of the main aims of synthetic biology research. Implementing this recent technology with optogenetic approaches amplifies further the toolbox of engineered systems, allowing for elevated spatial and temporal control of (d)Cas9 variants with high versatility (Fig. 3j).[20,121]

## Nanogenetics: a novel strategy for controlling molecular mechanisms?

Nanomedicine offers concrete approaches to improve the delivery and targeting of biologically active compounds. By contrast, synthetic biology triggers remote and highly controlled activation of processes at the subcellular level. Consequently, combing nanotechnology with synthetic biology approaches could allow for the design of alternative and unconventional solutions. Here, we introduce the concept of 'nanogenetics', which refers to a new discipline aiming to incorporate stimuli-responsive nanoplatforms into genetic circuits designed by using synthetic biology approaches. These evolved bio-based tools with potentiated abilities could be able to match the elaborateness of natural systems while being regulatable at distance by the use of specific stimuli.

The design principle of nanogenetics exploits the concept of modularity. Modularity is a well-known concept in biological systems and can be defined as a group of independent subunits that interact to increase the efficiency of a common network.[122] Biological modularity becomes evident, for instance, when considering a cell that needs to interact constantly with its continuously changing environment to survive. Furthermore, to assure high performance, it must not only capture diverse and transient external inputs, but also be able to reprogram its behaviour accordingly.[123] A synthetic switch designed according to the principles of biological modularity can be defined as a two-component system: a sensitive element responsible for capturing selectively an external signal, and a coupled transducer module, relevant to its subsequent forwarding in the form of a specific output.[124] Indeed, both artificial and natural systems using biological modularity, should: (i) recognize a specific input; (ii) transduce the obtained message; and (iii) process it by producing an adequate response.[125] Provided that these single components have been well characterized both singularly and as a complex, the behavior of the system can be easily interfered with, thus predicted and even controlled.[126] To avoid the interaction of the synthetic modules with native ones, it is clear that extensive studies of all components must be carried out *a priori* to ensure efficient decoupling and avoid unwanted interactions (orthogonality principle).

Synthetic biology approaches allow for the design of a variety of tailor-engineered modules that can be combined at will. The major current limitation of synthetic biology results from the lack of many stimuli-responsive modules (e.g., temperature, light, or chemical-inducible protein domains) or the poor orthogonality of existing ones. Implementing nanotechnologies

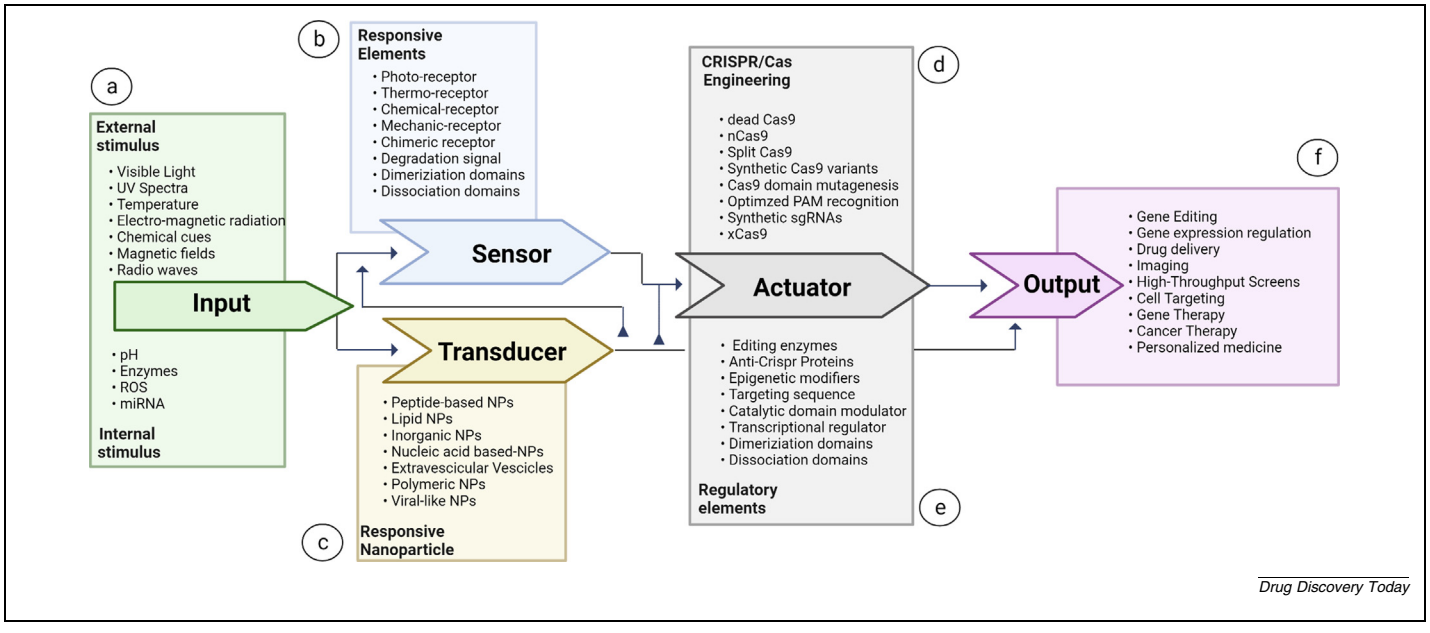


**FIGURE 4**

Schematic of a proposed nanogenetic approach for the construction of a CRISPR/Cas switch. An input of a distinct entity (a) is perceived by a sensitive element (b, c). The interaction of these leads to the creation of a nanosensor with specific chemophysical properties. Re-engineering of natural components by synthetic biology approaches (d, e) allows for the creation of a wide selection of interactive actuator modules. The cooperation of a nanosensor with the CRISPR/Cas toolbox leads to the formation of a nanotransducer characterized by a customized output (f).

in the design of a synthetic switch would allow the number of available modules to be expanded and, consequently, the ability to rationally engineer the whole system. Both the sensor elements and signal transducers could benefit from nanotechnology by exploiting its great variety of chemophysical and biological properties. Nanomaterials could respond to an internal or external signal to 'switch' the delivery or the activity of the gene editing machinery ON or OFF. pH-sensitive systems are widely used to release a therapeutic cargo in specific organs where pathological conditions, such as cancer and inflammation, cause a change in the pH.[127] An alternative approach is exploiting the reducing environment of tumors as a unique input signal that induces the degradation of redox-responsive nanocarriers in tumor cells and the delivery of the therapeutic load.[128] Similarly, a tunable drug delivery system can be generated by using nanocarrier molecules that are substrates for enzymes, such as proteases, glycosidases, and phospholipases, which are aberrantly expressed in specific tissues under pathological conditions.[129] Tunable delivery of therapeutics can be further induced by external stimuli by using, for instance, nanocarriers that are sensitive to non-ionizing radiations (visible, ultraviolet, and infrared light, static magnetic field, micro-, and radio waves, etc.) or ionizing radiations. Here, the stimulus induces the generation of thermal energy and/or physical forces that can cause the release of a specific drug.[130] The advantages of these tunable systems are, among others, the spatiotemporal control of therapeutic delivery at a specific site, noninvasiveness, and tissue penetration depth.

In addition, the nanomaterial could act as a contrast agent that amplifies the input signal or as a 'nanotransducer' itself, which converts a signal into another form of energy that could be further exploited by a stimuli-responsive protein domain. Rationally designed proteins demonstrate the ability to switch between different folding topologies [19] and the conformational change is conditioned by reaching the energy gap between the two folds.[18] In this context, stimuli-responsive nanomaterials can be understood as local nanogenerators of energy that can be used to activate or disengage Cas variants that have been screened to be energy activatable.[131] The implementation of nanomaterials in the design of a synthetic switch would increase not only its responsiveness, but also its output selectivity and sensitivity.[132] Another important advantage of nanotechnology is given by its low-cost and simple handling, creating a good basis for the design of CRISPR/Cas off-the-shelf nanoformulation-based therapies, potentially improving accessibility to the technology in the future. For this purpose, we introduce the concept of a CRISPR/Cas-switch, designed by a nanogenetic approach (Fig. 4).

Here, an external or internal input (Fig. 4a) is sensed by the responsive element (Fig. 4b) that often comprises a protein domain undergoing a conformational change in response to the stimulus. This shift can be efficiently triggered by a stimuli-responsive NP that amplifies or transduces the input in the signal necessary for driving the change (Fig. 4c). The conformational change activates the actuator, rationally engineered with the elements of the CRISPR/Cas toolbox (Fig. 4d, e). Indeed, a specific output is generated (Fig. 4f) by coupling the effector protein (Fig. 4e) that modulates gene expression to the effector nanocarrier (Fig. 4c), which targets a specific locus of the regulation. Specific targeting is further possible by exploiting the tunable drug delivery properties of the nanomaterial.

Moreover, optical, thermal, or magnetic inputs, for instance, are non-invasive and can be highly regulated, enabling systemic activation and intracellular guidance of CRISPR/Cas-equipped nanostructures even at a single cell level, within a complex environment such as a living organism. The necessary level of specificity is further granted by the computational design of the system, obtained by the coupling of synthetic regulation with nanotechnology (Box 1).

Box 1 Engineering of a tunable nanogenetic CRISPR/Cas9 platform.In the nanogenetics approach, a CRISPR/Cas9 switch is based on the rational design of stimuli-responsive nanoplatforms. From a biochemical perspective, this engineered platform is optimized to gain specific features, such as crossing biological barriers, improving cell penetration, targeting specific cell types, or inducing endosomal escape. From a physical point of view, the nanoplatform design is optimized to sense an external or internal input (e.g., a change in pH or temperature, a magnetic or electromagnetic field) and, eventually, to amplify or transduce a signal for the activation/disengagement of the gene-editing activity (e.g., by inducing a conformational change in a stimuli-responsive protein domain). The tunability of the CRISPR/Cas9 switch increases its safety profile. Notably, it also represents a nonviral strategy for *in vivo* delivery.

## Concluding remarks and perspectives

The research accumulated to date indicates how a nanogenetic approach could advance the CRISPR/Cas toolbox, as it focuses on the design of a nanotechnology-equipped synthetic tool. This novel technique can be easily regulated at different scales because of its modular structure, allowing it to overcome major limitations encountered with traditional genome-editing techniques. An essential part of transitioning from research to the clinic is the achievement of safe genome editing; that is, the ability to precisely edit a specific genetic locus without any adverse perturbation in a defined cellular target. Although the development of sophisticated and versatile CRISPR/Cas variants and tools, which are continuously evolving, marked the beginning of a new scientific chapter, the process is only in its early stages. Modern genome-editing holds great potential for disease prevention and treatment but still requires optimization before being applied widely *in vivo*. In this context, two emerging sciences that characterized the past few decades might offer a solution. Nanotechnology is increasingly used in biomedicine because it offers innovative solutions expected to improve therapeutics on different scales, for instance by acting as nanocarriers or by merit of the unique physiochemical properties of nanomaterials. By contrast, synthetic biology has become indispensable in most life science fields because it enables researchers to create and explore biological systems from scratch. Unifying these technologies into a hybrid one carries great potential for circumventing the constraints associated with them individually while

taking advantage of their best features. Here, we have discussed why the development and application of an innovative interdisciplinary technology, such as nanogenetics, is imminent within genome editing and might support major development in the field. The possibility to interact autonomously and dynamically with cellular systems while directing and/or reprogramming their evolution at distance using a nanogenetic approach together with CRISPR/Cas technology would enable faster diagnostics and drug discovery while benefitting at the same time preventive health applications and disease treatments. Thus, it is reasonable to believe that nanogenetics is conducive to the integration and expansion of CRISPR/Cas gene-editing technologies to the clinic while driving important advances in life sciences.

## CRediT authorship contribution statement

**Tiziana Julia Nadjeschda Schmidt:** Conceptualization, Writing – original draft. **Barbara Berarducci:** Conceptualization, Writing – original draft. **Soultana Konstantinidou:** Conceptualization, Writing – original draft. **Vittoria Raffa:** Conceptualization, Writing – original draft, Funding acquisition, Resources, Supervision.

## Data availability

No data was used for the research described in the article.

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## Declaration of interests

The authors declare no competing interests.

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