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In Situ Hybridization of Crispr Nanosponge - Hydrogel System for Localized Gene Delivery

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Abstract: In situ hybridization (ISH) has long been an indispensable molecular technique for visualizing nucleic acids within their native cellular or tissue environments. By employing complementary probes that specifically bind DNA or RNA sequences, ISH enables precise detection of genetic material with chromogenic or fluorescent readouts. Recent advances, such as the hybridization chain reaction (HCR), have significantly improved the sensitivity and resolution of ISH, permitting robust analysis of low-abundance targets. Parallel to these developments, CRISPR/Cas9 has emerged as a transformative gene-editing platform capable of inducing targeted double-stranded DNA breaks and facilitating either gene disruption or precise repair. Together, these two technologies hold great promise for advancing both fundamental research and therapeutic applications.To fully realize CRISPR's potential, however, efficient and safe delivery strategies remain essential. Conventional vectors such as plasmid DNA, mrna, and ribonucleoprotein (RNP) complexes face limitations including instability, inefficient uptake, and risks of off-target activity. To address these barriers, nanocarrier systems—particularly nanosponges and hydrogels—have been investigated as versatile delivery vehicles. Nanosponges, with their porous three-dimensional architecture, enable encapsulation of both hydrophobic and hydrophilic molecules, offering controlled release and enhanced stability. Their biocompatibility and tunable properties make them attractive for encapsulating CRISPR/Cas9 components and protecting them from enzymatic degradation. Hydrogels, by contrast, provide an injectable, tissue-like platform capable of forming in situ and sustaining localized release of gene-editing complexes. Their structural versatility allows fine-tuning of mesh size, degradation kinetics, and swelling behavior, all of which influence CRISPR delivery efficiency. The integration of ISH, HCR, CRISPR/Cas9, nanosponges, and hydrogels represents a convergence of diagnostic precision with therapeutic innovation. ISH and HCR provide molecular tools for spatial mapping and sensitive detection of nucleic acids, while CRISPR/Cas9 delivers unparalleled editing precision. Embedding these tools within nanosponge and hydrogel carriers creates multifunctional systems capable of both localized imaging and gene modulation. This synergy opens new avenues in oncology, regenerative medicine, and infectious disease treatment, marking a significant step toward safe, efficient, and clinically translatable gene therapy.

Keywords: CRISPR, NANOSPONGES, HYDROGEL, gene delivery

1. Introduction

Gene therapy represents an advanced biomedical strategy that enables precise modifications of the genome, including targeted insertions, deletions, or base substitutions. This approach relies on nucleic acids, such as DNA or RNA, to treat, prevent, or potentially cure human diseases[1]. In situ hybridization has become a standard laboratory technique for analyzing gene expression and localizing specific DNA and RNA molecules within cells[2].Among the third-generation gene-editing technologies, the clustered regularly interspaced short palindromic repeats (CRISPR) system, coupled with Cas9 endonuclease, has emerged as the most prominent tool. CRISPR/Cas9 is highly flexible, efficient, and cost-effective, making it widely applicable across biomedical research and therapeutic interventions.To enhance the performance of such systems, hydrogels have gained significant attention as delivery platforms. Their three-dimensional, water-rich polymer networks provide biocompatibility, tunable properties, and the capacity for controlled and sustained release of biomolecules[3]. Injectable in situ hydrogels further offer advantages including biodegradability localized accumulation at tumor or diseased sites, reduced dosing frequency, improved patient compliance, and minimized systemic toxicity[1].Similarly, nanosponges have emerged as a promising nanoscale drug delivery system. These mesh-like colloidal structures can encapsulate a wide variety of therapeutic agents, enhancing the solubility of both hydrophilic and hydrophobic drugs. Their amphiphilic nature, resulting from hydrophobic internal cavities and hydrophilic external surfaces, enables efficient drug loading and controlled, prolonged release, thereby improving bioavailability[4]. By integrating nanosponges within hydrogel matrices, a hybrid system can be created that combines the high encapsulation and release-control capacity of nanosponges with the stability and localized delivery advantages of hydrogels. In situ hybridization of CRISPR-loaded nanosponge–hydrogel composites thus represents a promising strategy for localized gene modulation, with potential applications in regenerative medicine and targeted disease therapy.

2. Materials and methods

2.1 In situ hybridization

In situ hybridization (ISH) is a molecular technique used to detect and localize specific DNA or RNA sequences within cells, preserved tissue sections, or whole tissues. The method relies on the binding of a complementary nucleotide probe to the target sequence, enabling visualization of genetic material in its natural cellular or tissue environment[5]. In situ hybridization (ISH) is a technique that utilizes the specific binding of a labelled nucleic acid probe to complementary sequences in fixed cells or tissues[6]. The probe–target hybrids are then visualized using chromogenic, fluorescent, or electron microscopic methods. This method takes advantage of the natural base-pairing properties of nucleic acids and can be applied to DNA–DNA, RNA–DNA, or RNA–RNA interactions. Once

annealed, the labelled probe enables detection through isotopic or nonisotopic approaches, including fluorescent and nonfluorescent systems[5]. The in situ hybridization (ISH) procedure consists of several steps. First, a specific probe is designed and prepared, after which it is applied to either a native or fixed sample. The sample is then denatured to separate nucleic acid strands, followed by renaturation, allowing the probe to hybridize with its complementary target sequence—typically overnight. The probe, a short nucleic acid strand, binds specifically to the gene of interest. In the final step, the hybridized probe is detected using fluorescence microscopy or specialized imaging software. Complementary DNA strands can be separated by applying heat, altering salt concentration, exposing them to extreme ph, or introducing organic compounds such as dimethyl sulfoxide and formamide, all of which disrupt the hydrogen bonds holding the strands together. DNA denaturation is characterized by the hyperchromic effect, where UV absorbance at 260 nm increases as double-stranded DNA separates into single strands due to disruption of base stacking. This property enhances the sensitivity of DNA detection. After denaturation, the probe binds to the target DNA or RNA, followed by renaturation and overnight hybridization. The next day, a post-hybridization wash is performed to remove nonspecific binding and cross-hybrids, thereby minimizing background signals[7].

2.2 Preparation of tissue

Tissue preparation is an essential step in ISH and includes both fixation and permeabilization. Early ISH studies were mainly performed on frozen sections, obtained either by snap-freezing or by freezing after brief fixation (1–3 hours) in 4% paraformaldehyde with 0.1 M PBS (ph7.4), followed by cryopreservation in 30% sucrose. Frozen tissues preserve nucleic acids well but often display poor morphology due to freezing artifacts. Over the years, chemical fixatives have been tested, and today 10% neutral buffered formalin (NBF), the routine fixative in pathology, is regarded as the most suitable for ISH. To improve probe penetration, sections are commonly treated with proteases such as proteinase K[8]. Acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine further reduces nonspecific binding of charged probes. Using well-preserved cells is essential to avoid nucleic acid damage. Both frozen tissues and formalin-fixed samples, even those stored for several years, can be successfully used for ISH[5].

2.3 Probes

A probe is a single-stranded DNA or RNA molecule with a specific nucleotide sequence that enables it to recognize and bind to a complementary target sequence. This interaction is based on the principle of base-pair complementarity[7]. In in situ hybridization (ISH), various types of probes can be employed, such as synthetic oligonucleotides, complementary DNA (cdna), and complementary RNA (crna). The ideal probe length ranges from 50 to 300 bases. Common examples include double stranded DNA probes, single-stranded

antisense RNA probes (riboprobes), PCR-generated single-stranded DNA probes, as well as synthetic oligodeoxynucleotide and oligoribonucleotide probes[5].

2.4 Probe labelling and signal detection

Radioisotope labelling is a highly sensitive technique, though it is limited by the short half-life of isotopes, prolonged exposure times, and associated health risks[7]. Results can be quantified or semi-quantified using methods such as densitometry on film or silver grain counting, with hybridization sites visualized by autoradiography through Xray film or liquid emulsion. In contrast, nonisotopic labelling utilizes compounds like biotin, fluorescein, digoxigenin, alkaline phosphatase, or bromodeoxyuridine, which are visualized using histochemical or immunohistochemical methods[5].

2.5 Hybridization chain reaction

Hybridization Chain Reaction (HCR) is an enzyme-free, isothermal amplification technique known for its simplicity, efficiency, and versatility[9]. It combines features such as multiplexing, quantitation, sensitivity, and high resolution, making it applicable for the detection of both RNA and DNA. HCR is particularly useful for identifying mrnain cells, tissues, and even whole organisms. There are two main categories of HCR: linear and nonlinear, with nonlinear types further divided into branched, dendritic, and hydrogel-based clamped forms[7].

The mechanism involves two metastable nucleic acid hairpins, H1 and H2, each containing a toehold, stem, and loop domain. In the absence of an initiator, these hairpins remain kinetically trapped. When a cognate initiator strand is introduced, it triggers a sequential chain reaction in which H1 and H2 alternately open and assemble into long nicked double-stranded amplification polymers.

By integrating HCR initiators with molecular probes—such as nucleic acid probes, aptamers, antibodies, functionalized nanoparticles, and dnazymes—the method has been extended to detect a wide range of targets, including nucleic acids, proteins, and small molecules. Additionally, HCR cascades can produce diverse output signals such as fluorescence, chemiluminescence, bioluminescence, colorimetric signals, electrochemical impedance, electrochemical chemiluminescence, and energy dissipation[9].

To improve the detection sensitivity of low-abundance targets in cancer cells, nucleic acid amplification methods are commonly applied. A notable example is the hmns/ACD system, which integrates hybridization chain reaction (HCR) with autocatalytic dnazymeamplification. In this system, four HCR hairpin probes and a dnazymesubstrate are assembled onto a honeycomb mno_2 nanosponge (hmns). When exposed to glutathione (GSH) and the target microrna mir-21, both HCR and dnazyme catalysis are initiated, leading to a strongly amplified fluorescence signal. This strategy offers high sensitivity, excellent resistance to interference, and enables fluorescence imaging within specific cellular organelles through DNA-functionalized nanomaterials[10].

3. CRISPR/CAS9

Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/Cas9 is a groundbreaking genome-editing tool that has revolutionized biomedical research. It allows precise correction of genetic mutations and enables regulation of gene expression in both cells and whole organisms in a fast, cost-effective, and efficient manner. This system is widely applied in generating cellular and animal models, conducting functional genomic screens, and visualizing genomes in real time. Experimental studies have shown its ability to repair defective DNA in mice, effectively curing genetic disorders, and its potential application in modifying human embryos has also been reported. Clinically, CRISPR/Cas9 holds significant promise in gene therapy, treatment of infectious diseases such as HIV, and engineering patient-derived cells for cancer and other disease interventions[11].

3.1 Overview of CRISPR/CAS9

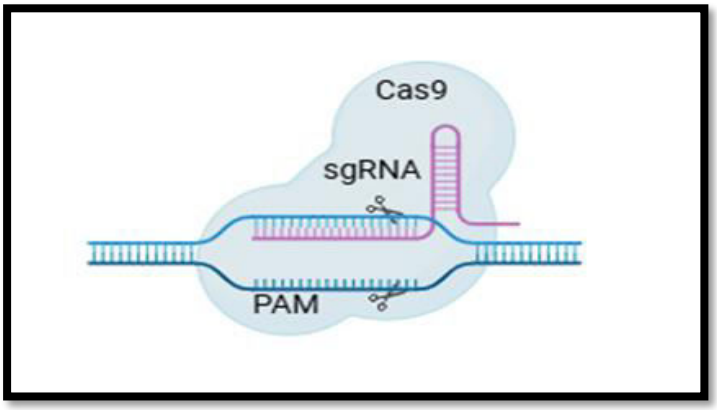


Figure 1 The CRISPR/Cas9 system.

CRISPR/Cas9 is a genome-editing tool that relies on two main components: a guide RNA, which directs the system to a specific DNA sequence, and Cas9 (CRISPR-associated protein 9), an endonuclease that generates a double-stranded DNA break to facilitate targeted genetic modifications[11].

3.2 Mechanism of CRISPR/CAS9

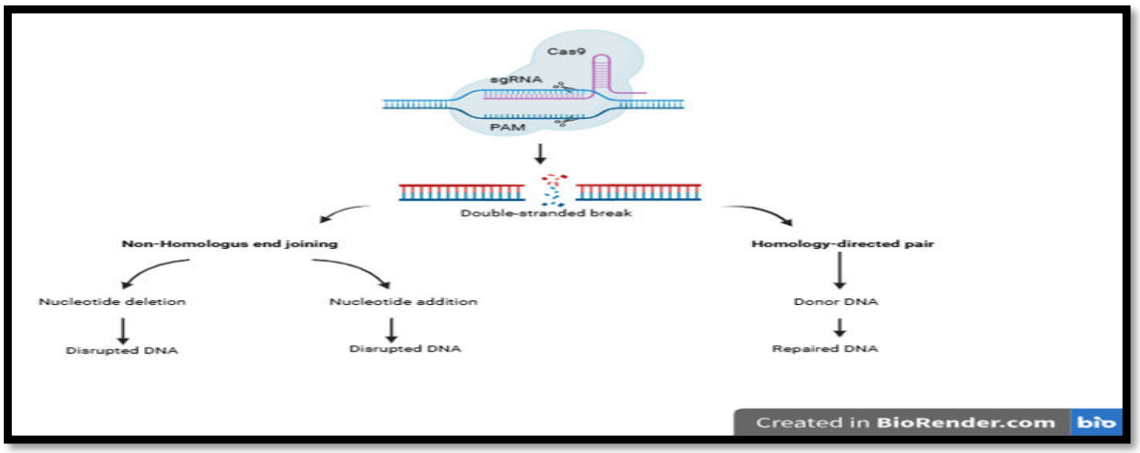


Figure 2: This figure illustrates the CRISPR-Cas9 genome editing process, highlighting the role of Cas9 and sgrnain creating a double-stranded break in DNA at a specific target site. Following the break, the repair mechanisms are depicted, showing two pathways: non-homologous end joining, which can lead to nucleotide deletion or addition and result in disrupted DNA, and homology-directed repair, which can utilize donor DNA to create repaired DNA.

CRISPR was first discovered in Escherichia coli in 1987 and is categorized into two major types with 16 subtypes, based on the recognition and cleavage mechanisms of endonucleases. Among these, the Type II CRISPR/Cas9 system is the most widely applied in mammalian cells owing to its high precision. This system comprises three essential components: CRISPR RNA (crrna), trans-activating CRISPR RNA (tracrrna), and the Cas9 endonuclease. The crrnacarries a 20-nucleotide protospacer sequence for target recognition along with a region that binds tracrrna. The tracrrnaassociates with crrnaand recruits Cas9 to assemble the ribonucleoprotein (RNP) complex. To streamline the process, crrnaand tracrrnaare often fused into a single guide RNA (sgrna), which enhances Cas9 targeting efficiency. Recognition of the target requires the crrna5′ end to bind a sequence adjacent to a protospacer adjacent motif (PAM), typically "NGG," which enables discrimination between foreign DNA and the host genome. Cas9 contains six domains, with the HNH domain cleaving the complementary DNA strand and the ruvcdomain cleaving the non-complementary strand. The resulting double-strand break (DSB) is repaired either by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ directly ligates DNA ends, frequently introducing insertions or deletions (indels) that disrupt gene function, resulting in gene knockout. Conversely, HDR employs a donor DNA template for precise gene knock-in, though its efficiency is limited as it occurs only in dividing cells[12].

3.3 CRISPR-Based Gene-Editing Tools

CRISPR technology enables precise gene editing in eukaryotic cells. Studies on Cas9 have produced functional variants and derivative tools through targeted modifications, while the discovery of other Cas proteins has broadened the scope of editable genes. To improve efficiency and safety, researchers have also developed specialized vectors for the delivery of CRISPR components into cells and organisms.

3.4 Composition of CRISPR/Cas9

3.4.1 Sgrna

The single guide RNA (sgrna) is the key component that directs Cas9 to its target sequence. In the natural CRISPR system of bacteria and archaea, fragments of invading genetic material such as phages or plasmids are stored within the CRISPR array as spacers. When re-exposed to the same invader, the array is transcribed into precursor CRISPR RNA (pre-crrna), which pairs with a trans-activating CRISPR RNA (tracrrna).This precursor undergoes two processing steps:

1. RnaseIII cleavage of the pre-crrna.

2. Cas9-mediated trimming to produce a mature crrna.

The mature crrnathen binds to tracrrna, forming a functional guide that directs Cas9 to the complementary DNA target. For practical use in eukaryotic systems, crrnaand tracrrnahave been fused into a single synthetic molecule—the sgrna. This sgrnarequires only a 20-nucleotide sequence next to a PAM site to specify almost any target site in the genome. Unlike zfnsand talens, CRISPR/Cas9 uses RNA-guided recognition, making precise sgrnadesign essential for efficient and accurate genome editing.

3.4.2 Cas9 Mechanism and Challenges

The sgrnadirects Cas9 to the target DNA, where cleavage occurs. In Streptococcus pyogenes Cas9 (spcas9), this process requires recognition of a PAM sequence positioned downstream of the target site, which triggers DNA strand separation. The seed sequence—the first 10 nucleotides of the crrnaadjacent to the PAM—base-pairs with the complementary DNA strand to initiate R-loop formation. Interaction of the PAM-distal DNA with Cas9's REC2 and REC3 domains further stabilizes and extends the R-loop. Once fully formed, Cas9's HNH and ruvcnuclease domains cut the complementary and non-complementary DNA strands, respectively.

However, wild-type Cas9 variants such as spcas9 and sacas9 are limited by several drawbacks, including off-target cleavage, chromosomal rearrangements, large deletions, and strict PAM requirements that constrain target site selection. To overcome these issues, engineered Cas9 versions have been developed to improve specificity and expand the range of editable genomic sites.

3.4.3 Plasmid DNA (pdna)

Pdnais a practical vector for CRISPR delivery because it is stable, easily modified, and can be produced in large amounts. After entering the cell, it reaches the nucleus with the help of nuclear localization signals (NLS) and transcribes mrnafor Cas9 and sgrna, though this process is relatively slow. Delivering CRISPR/Cas9 as mrnacan speed things up, but mrnais unstable, degrades quickly, and requires very long sequences when Cas9 is fused with effector proteins, limiting its application. Cas9 ribonucleoproteins (rnps), pre-assembled from purified Cas9 protein and sgrnain vitro, work immediately once inside the cell and provide rapid, transient activity. However, their delivery is difficult due to their large size, charge, and complex structure, often requiring electroporation or cell-penetrating peptides. More recently, exosomes have been identified as a promising natural system for delivering Cas9 rnps, offering an efficient and biocompatible alternative[13].

4. Formats and Key Factors of CRISPR/CAS9 Systems

The CRISPR/Cas9 system requires both the Cas9 protein and the single-guide RNA (sgrna) to achieve gene editing. Effective application depends on the successful delivery of these components, which can be accomplished through various formats tailored to experimental or therapeutic needs. Selection of an

appropriate delivery vector must consider factors such as the size and charge of the cargo, while ensuring high targeting precision and editing efficiency remains critical for optimal outcomes.

4.1 Formats of CRISPR/Cas9 systems

Delivery formats and payloads	**Mechanism**	**Advantages**	**Disadvantages**
DNA(Plasmid encoding Cas9 + sgrna)	Plasmid enters nucleus → transcribed to mrna→ translated into Cas9 protein + sgrna.	Simple, stable, and easy to store. Convenient for large-scale handling.	Delayed action (needs transcription/translation). Nuclear delivery is inefficient. Risk of plasmid integration into genome. Higher off-target activity, lower efficiency.
Mrna(Cas9 + sgrna)	Mrnadelivered to cytoplasm → directly translated into Cas9 protein → binds sgrna.	Rapid gene editing. No integration risk. Reduced off-target effects vs DNA.	Naturally unstable, degrades quickly. Requires chemical modification for stability and efficiency.
Protein(Cas9-sgrna RNP complex)	Pre-assembled Cas9–sgrnaribonucleoprotein (RNP) delivered directly into cells.	Immediate genome editing. No genome integration risk. Rapid Cas9 degradation → less immune response & toxicity. Highest precision, minimal off-target effects.	Production and purification are costly and complex. Limited stability during storage and delivery.

Table 1 Overview of CRISPR/Cas9 Deliver methods

4.2 Key Factors in CRISPR/Cas9 Delivery

4.2.1 CRISPR/Cas9 Loading

Loading CRISPR/Cas9 components is challenging because of the large molecular size of plasmids, mrna, and the Cas9 protein. While physical methods can bypass size restrictions, viral vectors are limited by their packaging capacity, especially for plasmids exceeding 4 kb. In addition, the positive charge of native Cas9 reduces compatibility with cationic carriers, requiring chemical or structural modifications of either the protein or the carrier. Naked plasmids, mrna, and proteins also face rapid enzymatic degradation and immune clearance in circulation. To overcome these obstacles, nanocarriers with polyethylene glycol (PEG) coatings or stimuli-responsive designs have been developed to enhance stability, extend circulation time, and improve delivery efficiency.

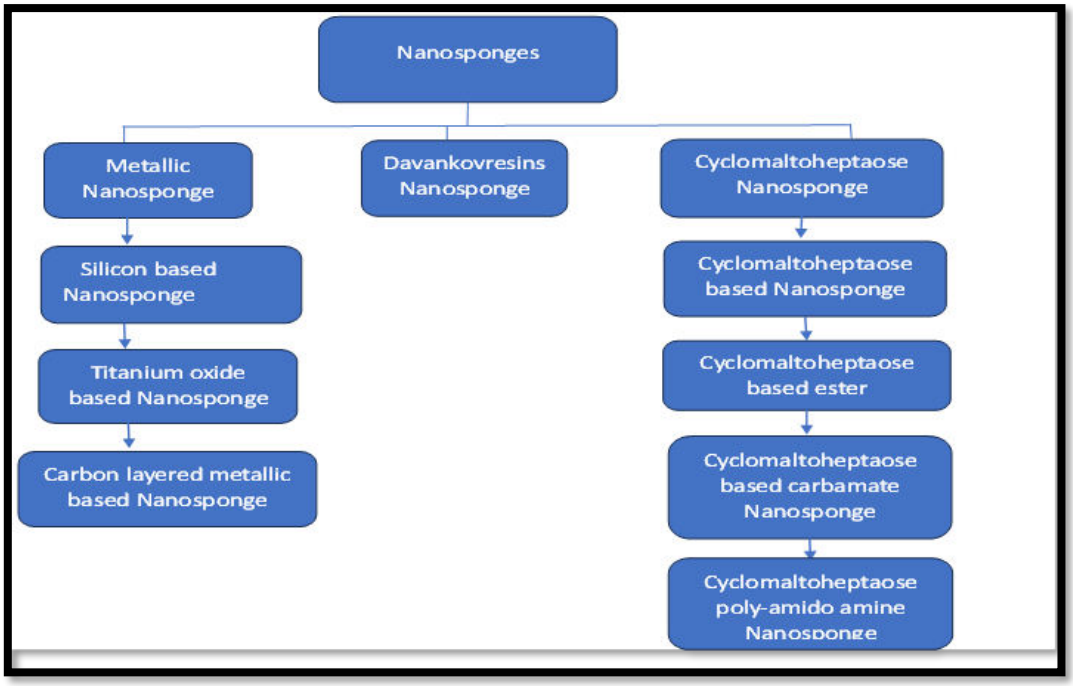
4.2.2 Targeted CRISPR/Cas9 Delivery

To overcome delivery barriers, researchers have engineered nanocarriers with polyethylene glycol (PEG) coatings and environment-responsive features that enhance both stability and efficiency. Improving targeting and delivery strategies is essential to fully realize the potential of CRISPR/Cas9 in genome editing and to broaden its clinical applications. Although viral vectors achieve high transfection efficiency, their use is limited by the risk of random genomic integration. Non-viral vectors, on the other hand, are increasingly preferred for their safety, design versatility, and scalability. Recent approaches include ligand-functionalized chitosan nanoparticles and extracellular vesicles, which achieve higher specificity through receptor-mediated binding. Additionally, stimuli-responsive nanocarriers activated by intracellular cues—such as ph, ATP, or glutathione—enable controlled and precise gene editing. Externally regulated systems, including light- and magnetic-responsive nanoparticles, further expand opportunities for safe, tunable, and efficient CRISPR/Cas9 delivery[14].

5. Nanosponges

Nanosponges are an innovative class of hyper-crosslinked polymer-based colloidal structures composed of solid nanoparticles containing nanosized cavities. They enhance the solubility of poorly water-soluble drugs and active constituents. Owing to their inner hydrophobic cavities and outer hydrophilic branches, nanosponges can encapsulate both hydrophilic and hydrophobic drug molecules, providing exceptional versatility[15]. Typically ranging in size from 1 to 1000 nm, they are engineered to encapsulate and deliver drugs or other active agents in a controlled and targeted manner[16]. These systems are fabricated from biocompatible and biodegradable polymers—such as hyper-crosslinked polymers or other crosslinked materials forming a three-dimensional network[17]. Nanosponges can be administered via oral, parenteral, topical, or inhalational routes, making them a highly adaptable platform for advanced drug delivery[18].

5.1 Types of NANOSPONGES



5.2 Components Used in the Preparation of NANOSPONGES

The preparation of nanosponges involves several essential components, including polymers, crosslinking agents, copolymers, and polar solvents. Together, these constituents form the porous, sponge-like architecture of the nanosponge, enabling efficient encapsulation and controlled release of therapeutic agents[19]. The degree of crosslinking is a critical parameter, as it is directly influenced by the concentration of crosslinkers used. This, in turn, significantly affects both the drug encapsulation efficiency and the release profile[20].

Polymer	Copolymer	Cross Linkers	Polar Solvents
Methyl beta cyclodextrin	Ethyl cellulose	Di-aryl-carbonates	Dimethylformamide
Hyper crosslinked polystyrene cyclodextrin (alkoxy carbonyl cyclodextrins)	Poly(Valero lactone allyl Valero lactone)	Carbonyl diimidazole	Ethanol
Hydroxy propyl beta cyclodextrin	Polyvinyl alcohol	Dichloromethane	Dimethylacetamide

Table 2 - The specific compounds employed in the preparation process

5.3 Advantages

Enhanced stability, aesthetic appeal, and formulation flexibility.

Non-mutagenic, non-irritating, non-toxic, and biodegradable.

Cost-effective and easy to scale up for industrial production.

Water-soluble nature allows encapsulation within nanosponges with the aid of adjuvant reagents.

Provides predictable and controlled drug release.

Masks unpleasant flavors, improving patient compliance.

Particle size can be tailored by adjusting the crosslinker-to-polymer ratio.

Facilitates commercial-scale manufacturing.

Improves therapeutic index and prolongs duration of action.

Enables efficient entrapment of active ingredients with reduced side effects.

Supports extended release, offering continuous action for up to 12 hours.

Exhibits thermal stability up to 130 °C[15,16,18].

5.4 Disadvantages

Performance depends on the loading capacity and drug release rate.

More suitable for smaller molecules than larger ones.

Crystallization within nanosponges can reduce loading capacity and overall performance.

Different para-crystalline forms exhibit varying loading capacities.

Primarily applicable to small molecules.

Challenges in scaling up while maintaining uniform properties.

Some drugs may exhibit unpredictable release kinetics.

Limited stability for sensitive drugs such as enzymes or peptides.

Higher production costs compared to conventional formulations[16,18].

5.5 Characteristic Features of Nanosponges

Offer a wide range of particle sizes (in micrometers or smaller) with tunable cavity polarity.

Particle size can be precisely controlled by adjusting the crosslinker-to-polymer ratio.

Non-toxic, porous particles that are insoluble in most organic solvents and thermally stable up to 130 °C.

Remain stable within a phrange of 1–11.

Drug-loading capacity is influenced by the degree of crystallization.

Form clear or opalescent suspensions in water.

Exist in paracrystalline or crystalline forms, depending on processing conditions; the crystal structure plays a key role in drug complexation.

Paracrystallinenanosponges can display varied drug-loading capacities.

Can be regenerated through simple thermal desorption, solvent extraction, microwave treatment, or ultrasound methods.

The three-dimensional architecture enables capture, transport, and selective release of diverse substances.

Can be targeted to specific sites due to their ability to bind with various functional groups.

Form both inclusion and non-inclusion complexes when combined with different drugs[15,20].

6. Drug Release from Hydrogel Matrices: Mechanisms and Processes

Hydrogels are hydrophilic mixtures that uniquely combine the properties of solids and liquids. Their structure is based on a three-dimensional network of randomly cross-linked macromolecules. Within this structure, three phases coexist: the polymeric solid matrix, the interstitial fluid, and the ionic phase. The solid matrix is made up of cross-linked polymeric chains that trap water and biological fluids, giving hydrogels their characteristic elasticity and tissue-like properties. The ionic phase, containing mobile ions and counterions, contributes to the swelling behaviour due to the presence of electrolytes[21].

Hydrogels can be derived from both natural and synthetic polymers. Natural hydrogels often show limitations such as weak mechanical strength or immunogenic responses, while synthetic versions lack intrinsic bioactivity. Nevertheless, both offer valuable advantages, including biocompatibility, biodegradability, and suitability for drug delivery applications. Drugs can be incorporated into hydrogels either after gel formation (post-loading) or during gel formation (in-situ loading), and release behaviour depends on diffusion, swelling, reversible interactions, or covalent bond degradation[21].

The geometry of a hydrogel-based delivery device significantly influences drug release kinetics. These devices can take the form of thin films, spheres, cylinders, or irregular solids[21]. The nanostructure of a hydrogel is described by three key parameters: the polymer volume fraction in the swollen state, the molecular weight between cross-links, and the network mesh size. These parameters control the diffusion rates of molecules within the swollen network[22].

The average molecular weight between cross-links indicates the degree of polymer entanglement and can be estimated using the Flory-Rehner equation[22]. The mesh size, representing the accessible space for molecular movement, defines whether a hydrogel is macro porous, microporous, or nonporous. Drug diffusion is directly influenced by these structural features, as shown in swollen and deswollenstates[21].

6.1 Mechanism of Drug Release

The release of drugs from hydrogels is governed by several physical and chemical phenomena, depending on hydrogel type, preparation technique, device geometry, and environmental conditions. These include:

1. Wetting of the device surface by the release medium.
2. Penetration of the medium into the hydrogel pores.

3. Degradation of drug and/or polymer.
4. Diffusion of degradation products and drug molecules through the matrix.
5. Ph changes within the hydrogel due to degradation.
6. Polymer swelling or pore closing.
7. Osmotic effects and hydrostatic pressure changes.
8. Adsorption/desorption processes.
9. Chemical reactions between drug and polymer degradation products.
10. Convection processes and shear forces.

From a process engineering perspective, drug release involves four fundamental steps: exterior diffusion, interior diffusion, desorption, and chemical reactions. These may be accompanied by shape changes (erosion, swelling, chain cleavage) and surface changes (reconstruction or chemical modifications)[22].

6.2 Exterior and Interior Diffusion

Drug diffusion can be divided into exterior and interior processes. Exterior diffusion occurs when drug molecules move from the hydrogel surface into the bulk medium. This process is described mathematically by mass transfer equations, where flux depends on surface concentration and mass transfer coefficients. Interior diffusion dominates most drug release processes and is typically explained using Fick's law. Drugs migrate through the internal pores of the hydrogel matrix, and two types of diffusion-controlled systems are described:
Reservoir devices, in which a polymeric membrane surrounds a drug-filled core. Here, diffusion across the membrane is the rate-limiting step. Matrix devices, in which drug molecules are dispersed throughout the hydrogel and diffuse outward over time[22].

6.3 Chemical Reactions

Drug release may also involve chemical reactions between the drug, polymer, and degradation products. These reactions occur within the hydrogel pores and may be reversible or irreversible, simple or complex, and fast or slow. Reaction products can undergo additional diffusion processes, influencing the overall release profile[22].

7. Shape Changes in the Hydrogel

7.1 Hydrogels often undergo shape modifications during drug release, including:

1. Chain cleavage, in which drug molecules covalently linked to the polymer backbone (prodrugs) are released following bond hydrolysis. The rate of cleavage depends on water diffusion and bond stability.
2. Matrix erosion, controlled by water penetration into the hydrogel and degradation of polymer backbones. Gopferich's theory of polymer erosion distinguishes between bulk erosion (diffusion-limited) and surface erosion (reaction-limited).

3. Swelling, a key mechanism in which the hydrogel transitions from a glassy to a rubbery state. In this swollen state, drug molecules diffuse more freely, and the release rate depends on the gel swelling speed.

The balance between water diffusion and polymer degradation can be expressed as an erosion number, which predicts whether bulk or surface erosion dominates[22].

7.2 Swelling-Controlled Release

Hydrogel swelling is a crucial factor in drug release, particularly when the diffusion of the active agent is slower than the swelling process. A swelling-driven phase transition occurs when the glass-rubber transition temperature of the polymer is exceeded, dividing the hydrogel into distinct swollen and glassy regions[22].

In such systems, two main factors influence drug release: the diffusion path length of molecules (which reduces concentration gradients) and the mobility of polymer chains (which increases relaxation rates). The Deborah number (De) is used to characterize swelling-controlled release. For

Small values (De << 1), drug release follows Fickian diffusion, while for large values (De >> 1), swelling dominates.

Peppas and Sahlin proposed a modified empirical power law to describe swelling-controlled release, combining contributions from both diffusion and polymer relaxation:

$M_t/M_\infty = k1*t^m + k2*t^{(2m)}$

Where,

M_t/M_∞ is the fraction of drug released at time.

K1 and k2 are the kinetic constants.

M correspond to diffusional exponentrelated to geometry of the release system.

T is the time[22].

8. Hydrogel-Based Platforms for Localized CRISPR Gene Delivery

1. Thermosensitive Polyamine-Modified Hydrogel for Melanoma Treatment

A thermosensitive, polyamine-modified injectable hydrogel (Psh) was developed for localized co-delivery of CRISPR/Cas9 ribonucleoproteins (rnps) targeting YB-1 and the chemotherapeutic agent doxorubicin. Once injected into melanoma tumors, the hydrogel formed in situ, enabling sustained drug release, efficient cellular uptake, and high gene-editing efficiency (up to 53% indel rate) both in vitro and in vivo. This targeted delivery system not only minimized off-target effects but also enhanced the synergistic therapeutic response when combined with chemotherapy[1].

2. Hydrogel–Nanofiber Composite Scaffold for Localized CRISPR Activation in Wound Repair

A biocompatible hydrogel/nanofiber (Gel/NF) composite scaffold was developed to deliver non-viral crispracomplexes in a controlled manner. In a rat

skin defect model, the scaffold enabled localized and sustained gene activation, leading to increased VEGF expression and accelerated wound healing[23].

3.Layer-by-Layer Self-Assembling Peptide (lbl-SAP) Nanofiber Scaffolds

Electrospun nanofibers were coated with self-assembling peptides using a layer-by-layer technique to immobilize CRISPR/dcas9 plasmids. This system provided localized and sustained gene delivery while supporting cellular adhesion. Activation of the GDNF gene in proof-of-concept studies enhanced neurite outgrowth, underscoring its promise for neural tissue engineering[24].

4.Liposome-Templated Hydrogel Nanoparticles (lhnps) for Systemic CRISPR Delivery

Lhnpsare constructed with a polyethylenimine (PEI)-based hydrogel core surrounded by a liposomal shell, enabling codelivery of Cas9 protein and sgrna. Unlike bulk hydrogels, they are suited for systemic administration, offering controlled release and efficient transfection. In tumor models, lhnpsachieved effective CRISPR-mediated PLK1 gene editing, underscoring their promise in cancer therapy[25].

9. Current affairs:

Smart Hydrogels – Hydrogels that respond to CRISPR activity (Cas9/Cas12/Cas13) or to local conditions (ph, ROS, enzymes) to release the gene-editing cargo only when needed.

DNA-Based Hydrogels – DNA gels act as both carrier and programmable scaffold, easy to load with CRISPR and naturally degraded in the body.

Safe Payloads (rnps) – More focus on delivering CRISPR as ribonucleoproteins (Cas9 protein + guide RNA) instead of plasmids, reducing long-term risks.

Nanosponge Carriers – Cyclodextrin nanosponges embedded in hydrogels protect CRISPR cargo and co-deliver drugs (e.g., chemo or immune agents).

Dual Delivery (Theranostics) – Hydrogels can both **treat** (gene editing) and **sense** (detect rnas/biomarkers) at the same site.

Injectable & 3D-Printable Gels – In situ gels that are syringeable, sprayable, or printable can conform to irregular tissues (tumors, wounds).

Combination Therapy – Hydrogels that co-deliver CRISPR with drugs or immunotherapy for stronger local treatment.

Targeted Adhesion – Bioadhesive gels (mussel-inspired, mucoadhesive) help keep the CRISPR system localized in specific tissues.

Layered Carriers – Hydrogels often carry nanoparticles (like lnpsor nanosponges) inside them, which then release CRISPR cargo into cells.

Built-in Safety – Some systems include ‘‘off-switches’’ like anti-CRISPR proteins or degradable linkers to stop editing if needed.

10. Discussion and conclusion

In situ hybridization serves as a precise technique for examining nucleic acid interactions within cells and tissues,offering critical insights into gene expression patterns and delivery outcomes. When integrated with CRISPR-based gene editing, nanosponge carriers, and hydrogel scaffolds, this strategy establishes a highly localized and well controlled platform for gene delivery. Nanosponge systems enhance the loading efficiency and stability of CRISPR components, while hydrogels enable sustained and sites specific release, thereby reducing systemic exposure and potential toxicity. Collectively, these elements improve the stability, specificity, and therapeutic effectiveness of genetic interventions.

Looking ahead, further progress will depend on optimizing hydrogel formulations, improving the biocompatibility of nanosponge carriers, and advancing CRISPR delivery technologies to ensure both safety and clinical applicability. Overall, the convergence of in situ hybridization with CRISPR–nanosponge–hydrogel systems represents a promising frontier for localized gene therapy and personalized medicine.

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