Review

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The Evolution of Tumor-Targeted Drug Delivery: From the EPR Effect to Nanoswimmers

Nour Zoabi[†], Adi Golani-Armon[†], Assaf Zinger, Maayan Reshef, Zvi Yaari, Dikla Vardi-Oknin, Zohar Shatsberg, Aseel Shomar, Janna Shainsky-Roitman, and Avi Schroeder*[a]

Abstract: Therapeutic nanotechnologies have made great progress over the past decade. Skepticism has been replaced by the understanding that precision at the nanoscale allows improved treatment modalities in humans. Principles for designing tumor-targeted drug delivery systems are described.

At first, the enhanced permeability and retention (EPR) effect was the major targeting mode, with up to 10% of the injected dose actually reaching tumors. To improve cellular uptake, sugars, antibodies, peptides or other ligands were added to the surface of nanotherapeutics. These can be coupled with external magnetic fields or ultrasonic waves to propel iron oxide or gas-filled particles towards the disease site.

Next-generation drug delivery systems will be capable of autonomously swimming towards the disease site and penetrating deep tissue, independent of blood or lymphatic flow. This has been shown to some extent with modified, drug-producing, bacteria. Interestingly, sperm may be nature's best example of a multifunctional, targeted, high-fidelity, self-propelled, delivery system that we can learn from.

Keywords: drug delivery · metastasis · nanoparticles · nanotechnology · personalized medicine

1. Introduction: Nanotechnology and Drug Delivery

Traditionally, medicine uses chemical or biological agents to fight diseases and relieve symptoms. Both systemic and local administration routes result in undesired interactions between the drug and healthy tissues. Advances in nanotechnology introduced sophisticated delivery systems for various medical applications, including drug carriers and personalized diagnostic tools.[1] Nanoparticles (Figure 1) are defined as synthetic constructs having at least one dimension between 1 and 1,000 nanometers long.[2] They are synthesized from a variety of materials including polymers, lipids, and metals, and can either encapsulate or be conjugated to their cargo.[3] Nanoscale drug delivery systems can be designed to have various desirable properties including size, charge, solubility, serum stability and bioavailability.

Surface modifications of drug carriers with targeting ligands are used to concentrate the drug at the disease site, increase therapeutic efficacy and reduce side effects.[4–7] Nanoparticles can be engineered to respond to external or internal stimuli, such as ultrasound, light, heat, enzymatic activity, or pH, as triggers for performing localized therapeutic tasks.[8–10]

In the field of cancer therapy, particulate nanocarriers are also valued for their promise to overcome multidrug resistance (MDR). Particles are taken up by cells via receptor-mediated endocytosis, thereby circumventing the cellular expulsion pumps.[11,12] Nanoparticle-based therapeutics are gaining acceptance in the clinic, with over 40 approved formulations and many others under investigation.[13,14] These systems are used for treating cancers, fungal infections, age-related macular degeneration, hepatitis, anemia, hypercholesterolemia, pain and many other conditions.[13,15]

2. Passive Targeting: How Efficient Is the EPR Effect?

The term 'targeting' is used broadly in the field of nanotherapeutics, usually implying injectable particles that reach a diseased tissue in an accurate manner. The enhanced permeability and retention (EPR) effect is the

[a] N. Zoabi, A. Golani-Armon, A. Zinger, M. Reshef, Z. Yaari, D. Vardi-Oknin, Z. Shatsberg, A. Shomar, J. Shainsky-Roitman, A. Schroeder
Laboratory for Targeted Drug Delivery & Personalized Medicine Technologies
Department of Chemical Engineering
Technion – Israel Institute of Technology
Haifa 32000 (Israel)
e-mail: avids@technion.ac.il

[†] These authors contributed equally

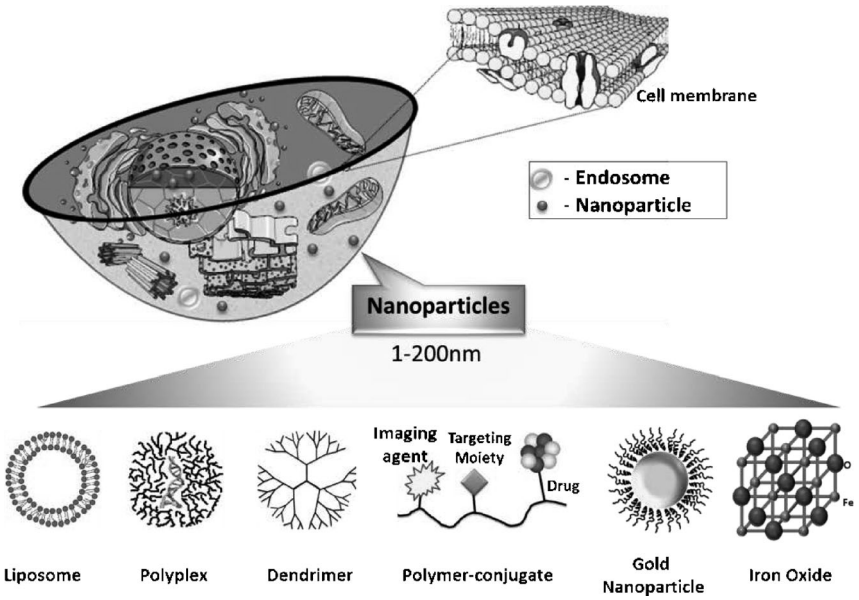

Figure 1. Nanomaterial platforms. Therapeutic nanomaterials have been designed to target cellular compartments within diseased tissues. The carrier can have one or more therapeutic functions, such as an imaging modality that is conjugated to a drug or an infrared-responsive material for ablation and sensing.

gold standard for nanoparticle targeting to sites of infection, inflammation and cancer.[16,17] In cancer, abnormal angiogenic processes contribute to the growth of imperfect and leaky tumor vasculature with pores in the range of 400 to 1000 nm.[18–20] Nanoparticles have been shown to extravasate through these pores, leaving circulation and entering tumors.[20,21] This process of extravasation and accumulation in tumors is known as the 'EPR effect' or as 'passive targeting'. Similar processes occur also during infection and inflammation.[16]

However, a closer look at the EPR targeting data reveals that only a small fraction (<10%) of the administered nanoparticles actually reach tumors (Table 1) or sites of inflammation.[22–26] In fact, the majority of the nanoparticles accumulate in the liver and kidneys.[27] Targeting can be improved by adding ligands to the surface of the nanoparticles or by physically propelling the nanoparticles towards the diseased tissue.[26,28]

Avi Schroeder is an Assistant Professor of Chemical Engineering at the Technion – Israel Institute of Technology. In 2012 he established the Laboratory for Targeted Drug Delivery and Personalized Medicine Technologies, which is focused on developing novel nanotechnologies for treating metastatic cancer. Avi is a Horev Fellow and an Alon Fellow as well as a former recipient of the Intel Nanotechnology, TEVA Pharmaceuticals, and the Wolf Foundation PhD Student Awards.

3. Active Targeting: Ligand-Based Nanoparticle Homing to Disease Sites

To enhance the therapeutic efficacy and reduce cytotoxicity, drug nanocarriers have been modified with targeting ligands that selectively bind receptors or molecules that are unique or overexpressed at the target tissues (Figure 2).[6] C-type lectin receptors (CLR) are a family of receptors that share structural homology in their carbohydrate recognition domain (CRD), where they bind common bacterial sugar molecules, thereby reporting bacterial infection. Several nanocarrier systems exploit this interaction, and by conjugating relevant sugar molecules were able to target CLR-expressing cells.[37] Notably, the affinity of ligand-CLR is significantly enhanced when a multivalent rather than a monovalent ligand is used.[38] Unfortunately, the synthesis of carbohydrate ligands and analogs, especially multivalent or complex carbohydrates, often requires many time-consuming, low-yield synthetic steps.[37] Moreover, many carbohydrate antigens are weakly immunogenic and lack specificity as they usually bind multiple receptors.[39] Finally, the affinity of carbohydrate ligands to their receptors is relatively weak, with dissociation constants in the low millimolar range.[40]

A frequently used alternative to carbohydrates are antibodies. The wealth of antibody-targeted systems under investigation provides a clear indication of their efficiency. Ibritumomab tiuxetan (Zevalin), brentuximab vedotin

Table 1. The EPR-based biodistribution of nanoparticles to tumor sites post intravenous administration.

Tumor type	Particle size and type	Targeting efficiency; % of the injected dose that reached the tumor site	Time post injection	Ref.
Sarcoma 180 (murine, subcutaneous)	70–160 kDa, proteins	7–8%	48–72 hr	[29]
Sarcoma 180 (murine, subcutaneous)	40–700 kDa, cationic polymer	3–4%	1–14 days	[30]
MDA-MB-231-H2N Breast cancer (mammary fat pad, murine)	80 nm polymeric micelle	5–7%	2–4 hr	[31]
J6456 lymphoma (murine, intra-peritoneal)	100 nm PEGylated liposomes	~10%	24–48 hr	[32]
C26 colon adenocarcinoma (subcutaneous, murine)	80–100 nm:		48 hr	[33]
	PEGylated	4%		
	Non PEGylated	2%		
Pancreatic adenocarcinoma (xenograft, murine)	38 nm, functionalized iron oxide	~4%		[34]
Human epithelial carcinoma (subcutaneous, murine)	135 nm gold nanorods	2%	24 hr	[35]
Human patients, 15 various solid tumors	90 nm PEGylated liposomes	0.5–4%	72 hr	[36]

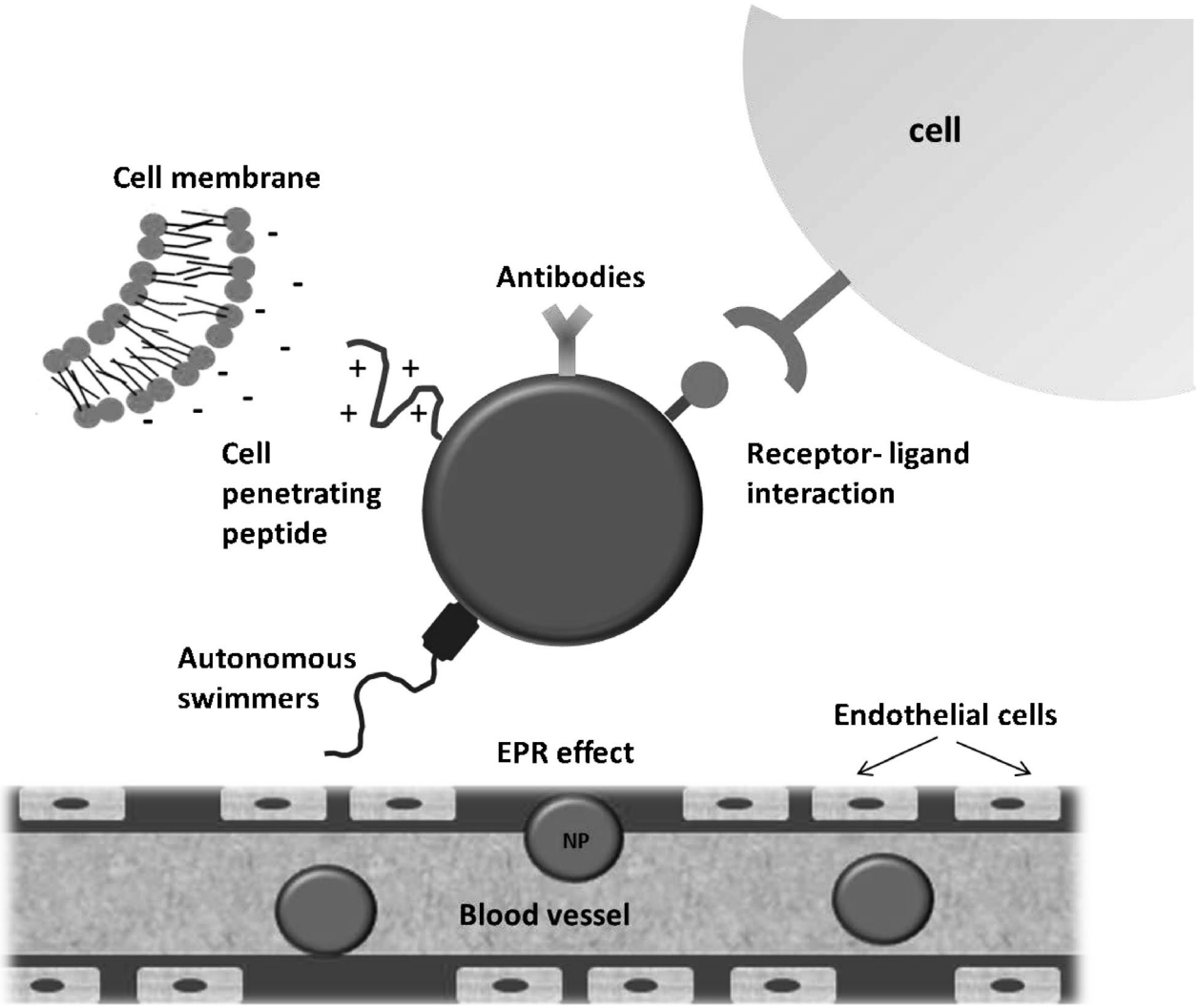

Figure 2. Targeting strategies. Nanoparticles can be targeted to diseased tissue via the enhanced permeability and retention effect. Once at the target site, several modes can be used to enhance deep tissue permeability, adhere to specific cells, and improve cellular uptake.

(Adcetris) and ado-trastuzumab emtansine (Kadcyla) are FDA-approved antibody-drug conjugates in clinical use. The limitations of antibodies, however, are their high cost and relatively large size. Moreover, even when humanized, antibodies may still elicit adverse immune responses and induce autoimmune side effects.[41]

Surprisingly, only 3.4% of 12 nm iron oxide nanoparticles, conjugated to the monoclonal antibody Herceptin, reached 3T6 murine tumors post intravenous administration.[42] Kirpotin et al.[43] and others[44] demonstrated that antibodies may not increase nanoparticle accumulation at the tumor site (which is dominated by the EPR effect); however antibodies increase cellular uptake at the tumor site significantly.

Peptide ligands are easily synthesized, elicit minimal to no immunogenicity, and in some cases were shown to bind cellular receptors with higher affinities compared to the corresponding carbohydrate ligand.[45] Nevertheless, peptide specificity to the target is usually lower than that of antibodies. Biomimetic and cell-based systems improve targeting. Mesenchymal stem cells, loaded with a drug or nucleic payloads, possess an intrinsic homing capacity towards wounded, regenerating or cancerous tissues.[46] Porous silicon nanoparticles functionalized with leukocyte membranes were shown to preferentially bind inflamed endothelium.[47] Platelet-mimicking particles home to tumor sites where they can recruit additional particles, resulting in a self-amplifying aggregation at the site of interest.[48]

4. Remote-Controlled Nanoparticle Targeting and Drug Release

External stimuli can improve targeting accuracy and control drug release profiles onsite. Magnetic fields have been used to attract iron oxide nanoparticles towards disease sites.[49–52] Propeller-like spiral particles (200–300 nm in width, 1–2 microns long) have been remotely driven using a homogeneous magnetic field.[53] At the disease site, an alternating magnetic field vibrates the nanoparticles, thereby elevating the local temperature and ablating diseased tissue.[54] When incorporated into temperature-sensitive nanoparticles, this thermal effect can be used as a means for triggering drug release intracellularly.[55]

Ultrasonic waves (acoustic streaming) have been used to steer gas-filled or metallic nanoparticles towards disease sites.[56,57] At the disease site, ultrasound can be employed to trigger the release of a drug from a nanocarrier using thermal or mechanical means.[58–60] Localized triggered drug release, at therapeutically relevant doses, has been shown to be superior to conventional treatment modalities.[8,10–12] Under low-frequency (<1 MHz) ultrasonic fields, cavitation, the growth and subsequent forceful implosion of gas bubbles, is generated. Cavitation is used to transiently permeate cells, thereby facilitating drug uptake.[61,62]

Light has been used to direct nanoparticle motion and coagulation;[63,64] ultraviolet (UV) rays were used to facilitate local adhesion of nanoparticles to cells, by releasing a cage molecule from the particle surface and exposing 'sticky' ligands.[65] Infrared light, which penetrates tissue to depths of ~1 cm,[66–68] can be used to heat gold nanoparticles after localizing in tumors.[69] These heating effects can be used to release drugs from temperature-sensitive particles or to ablate diseased tissue.[8]

5. Autonomous Swimmers as Drug Delivery Platforms

Micro and nanoswimmers – systems designed to autonomously navigate in situ while performing tasks – are entering the medical arena.[70] The great promise of these systems is the ability to detect malignancies and perform medical procedures with great precision. Autonomous therapeutic swimmers will be able to penetrate deep tissues independent of blood flow or lymphatic trafficking.[57,58]

This evolving field can be divided into synthetic and biological systems. While the principles of motion and activity are many times shared, the building blocks of each of these systems differ. This review focuses on the latter – therapeutic biological swimmers.

6. Bacteria as Targeted Drug Delivery Systems

Bacteria, the natural swimmers, provide many unique mechanisms for treating diseases that cannot be achieved with standard methods. Bacteria can actively penetrate tissue, target tumors, and controllably induce cytotoxicity.[71] They can deliver genes intracellularly, and have been used to ferry therapeutic nanoparticles and diagnostic agents to disease sites.[72–74] Most importantly, unlike other delivery systems, bacteria can multiply after reaching the disease site; therefore, even when only a small percentage of the injected dose targets the tumor, it can expand onsite and become therapeutically significant.[75]

Bacteria sizes range from 200 nm to above one micron, making them attractive for various drug delivery applications.[76] Bacteria have been used to regress tumors since the late 1800s[77–80] and are used to stimulate general or specific immune responses in vivo.[77,81–83] Bacterial therapies are appropriate candidates for specific targeting. It has been shown that several types of bacteria accumulate preferentially in tumors, including *Salmonella*, *Bifidobacterium*, *Escherichia* and *Clostridium*. Obligate anaerobes, such as *Clostridium* and *Bifidobacterium*, accumulate in hypoxic regions in the tumor,[84–86] while facultative anaerobes, such as *Salmonella* and *Escherichia*, can survive in

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both oxygenated and hypoxic environments. The latter have been shown to target tumors via multiple modes:[87–92] a) physical entrapment in the tumor's chaotic vasculature,[91,92] b) chemotaxis towards biomarkers that are secreted in the tumor microenvironment,[93–96] c) replication inside tumor tissue, and d) accumulation driven by the EPR effect.[93,95,97] It has been suggested that *Salmonella* propel towards elevated serine, aspartate and ribose levels in the tumor,[93–96] while others were leucine-arginine dependent.[95,96] Thereby, modified *Salmonella typhimurium* have been used to target and treat breast cancer.[96] Similarly, a non-pathogenic *E. coli* strain (M23) has been shown to grow preferably in the tumor microenvironment, due to elevated glucose and lactic acid levels in this tissue (Table 2).[73,75,98–100]

Table 2. Tumor pH decreases as the tumor volume increases (data sourced from references [116–119]).

Tumor type	Volume, cm^3	pH
Malignant melanoma (in human)	25	6.9
	100	6.6
Adenocarcinoma (in human)	100	7.2
	400	6.8
Squamous cell carcinoma (in human)	75	7.2
	300	6.4
Breast, lung (human xenograft in nude mice)	NA	6.8

Bacteria can also be the source of infection, inflammation and immune stimulation.[101] To address these issues, bacterial therapies must be designed to be sensitive to antibiotics. The development of *Salmonella* with altered lipid A in its envelope marked a major milestone in improving safety and reducing immune stimulation.[102,103] Another approach for reducing immunogenicity is to encapsulate bacterial machinery in non-immunogenic nanoparticles.[104,105] Such systems can be engineered to produce the desired protein therapeutic only at the target site, in response to an external trigger.[106,107] This onsite production approach will help reduce adverse effects of the drug to healthy tissues. Weighing the benefits of improved targeting and deep tissue penetration brought regulatory bodies to approve multiple clinical trials with bacterial cancer treatments (see also clinicaltrials.gov NCT01118819, NCT00004988, NCT00358397, NCT00623831, NCT00938080).[71,108–115]

7. Bacteria Produce Drugs Onsite

Bacteria are powerful microorganisms that can act as factories for producing a wide range of valuable drugs.[97] The bacteria can be alive, synthesizing recombinant proteins, or ghost vehicles, after removing all the cytoplasmic content. Bacteria have been used for treating viral diseases such as HIV and herpes, as immunogenic agents that increase cytotoxicity and as targeted delivery systems,[97,101] including cancer targeting and therapy.[120,121]

Clostridium and *Salmonella* are naturally cytotoxic and have been shown to regress tumors.[77–80] Bacillus Calmette-Guérin, a naturally occurring bacterium, is used for treating bladder cancer.[122,123] In this case, cytotoxicity is caused by sensitizing the immune system and competition for nutrients. Bacteria have been used to produce anticancer protein toxins,[121] either by directly expressing the toxin in the bacteria, or by delivering eukaryotic expression vectors to cancer cells.

Three primary categories of anticancer agents exist: cytotoxic agents that directly kill cancer cells, for instance diphtheria toxin (DT), *Pseudomonas aeruginosa* exotoxin A (PE) and cytolysin A; cytokines that stimulate immune cells to kill cancer cells, such as IL-2 and IL-18;[124–126] and tumor antigens that sensitize the immune system against cancer cells, such as RAF1.[127] Bacteria could be genetically manipulated to increase their effectiveness. For instance, bacteria can be engineered to express single-chain antibodies to inhibit proteins that are necessary for tumor cell function.[97,128]

7.1. Bacterial Motion at Low Reynolds Numbers

Bacteria have the ability to self-propel and sense the environment. Bacterial movement is facilitated by chemical stimuli or chemotaxis.[129] Chemotaxis activates protein cascades within the bacteria that eventually drive flagellar motion.[130,131] Both *Salmonella* and *Escherichia* have 4–6 flagella on their outer bacterial wall.[132] Flagella are made of the protein flagellin and enable swimming forward by counter-clockwise motion.[133]

Bacterial flagella enable tissue penetration and chemotactic receptors direct chemotaxis towards molecular signals. Chemotaxis denotes any change in the external environment of the bacteria (for example, changes in pH, temperature or nutrient concentration).[134–137] For instance, ribose and galactose receptors direct bacteria toward necrotic tumor regions, while serine receptors aid deep tissue penetration.[94] The combination of these receptors drives bacteria to specifically accumulate in tumors and migrate to distal tissues that are not accessible using traditional treatments.

Biological organisms on the scale of microns operate in a hydrodynamic regime in which inertia is negligible and motion is dominated by friction.[138] The Reynolds number, which represents the ratio of inertial to viscous forces (Table 3), is $Re \ll 1$, therefore each 'swimming stroke' propels the particle to a distance equal or shorter than the stroke itself. Cork screw helical strokes are common among biological and biomimetic swimmers as they avoid reciprocal strokes and maximize energy usage.[139–141]

Table 3. Typical Reynolds numbers for various swimmers in water.

Swimmer	Length, m	Velocity, m/s	Reynolds number
Dolphin	3	70	10^7
Human	2	10	10^4
Goldfish	0.05	0.2	10^2
Larva	0.004	0.02	10
Bacteria	0.000001	0.00001	10^{-9}

When an object, with characterized linear dimension L, moves through a fluid with a relative velocity V, the fluid's dynamic viscosity and density are μ and ρ respectively, and Reynolds number is defined as:

$$\frac{\rho \mathrm{VL}}{\mu} = \frac{\text{forces in fluid due to inertia}}{\text{forces due to viscosity (or friction)}}$$

The density and viscosity of water are: $\rho = 1_{kg/\ell}$; $\mu \approx 10^{-3}{}_{kg/(ms)}$.[142,143]

8. Sperm: Nature's Choice for Gene Delivery

When coming to design a new functional system, researchers often turn to nature for inspiration. In the field of drug delivery, particularly gene delivery systems, nature presents sperm as a multifunctional, sophisticated model that leaves man-made delivery systems far behind.

One of the limitations of synthetic, viral, or bio-inspired gene carriers is a restricted gene size to be delivered, as the DNA segment is cloned to a plasmid with a finite size. The human sperm, on the other hand, is able to carry 23 full chromosomes (half of the complete genome of a single somatic cell), at their native form.

Several mechanisms have evolved to enhance sperm stability and survival in the hostile environment of the female reproductive system. The blood–testis barrier (BTB) separates the testis from the circulation to block infiltration of antigens from the male blood to the semen,[144] as such antigens may elicit an immunogenic attack on the sperm once in the female reproductive system. To further repress any possible immunogenic response, the seminal plasma contains immune-response inhibitors that coat the sperm.[145] Moreover, the seminal plasma pH is slightly alkaline to protect the sperm from the acidic pH environment in the vagina.[146]

Unlike most synthetic delivery systems, sperm does not rely on the blood circulation for its passive motion towards its target, but is equipped with a motor-like organelle, a flagellum, empowered by multiple mitochondria, that provide it with a locomotion capacity.[147]

Another advantage of sperm as a delivery system is its excellent targetability. Where synthetic delivery systems only preferentially bind their target cells, sperm exclusively bind the egg, and cannot bind other cell types. While navigating from the vagina towards the fallopian tube, the sperm undergoes several processes resulting in the exposure of ligands to specific receptors on the egg. These ligand-receptor interactions are not only cell-specific but also species-specific, preventing inter-species fertilization.

In response to ovulation signals, sperm cells stored at the fallopian tube are hyperactivated and approach the tubal ampulla,[148] where ligand-receptor interactions between the egg and sperm trigger the release of sperm enzymes that perforate the egg membrane. The sperm content finally penetrates the egg, completing the last important step in gene delivery – DNA release.

These specialized properties make sperm the perfect gene delivery system to the egg; nevertheless, it cannot be easily translated into general gene delivery systems. Several unique characteristics of the sperm and scrotum present serious limitations for such applications. First, as sperm is intrinsically specific to the egg, its application as a delivery system for other cell types will require the incorporation of targeting moieties, thus complicating this simple and natural platform. The scrotum temperature is two to three degrees lower than physiological temperature, which is optimal for sperm development and survival. In vivo delivery applications necessarily involve physiological temperature, and are likely to damage the sperm. As mentioned above, sperm cells are separated from the circulation by the BTB and are therefore identified by the immune system as 'foreign' rather than 'self'. In vivo applications, especially without the protective activity of the seminal plasma, may result in a strong immunogenic response and sperm destruction.

In the future, the development of methods to endow sperm with temperature stability and immune tolerance would enable researchers to exploit this powerful system for a variety of applications.

9. Summary and Outlook

Advanced tumor targeting, in which a *majority* of the injected dose will reach the disease site and penetrate malignant cells, will require combining passive (i.e., EPR), active (ligands), and proactive (swimmers) modalities in a single nanoscale system. Translating nanotherapeutics into clinically approved drugs is not a simple task. Multifunctional nanotherapeutics pose new challenges to the regulatory bodies by introducing several risk factors simultaneously. Paradoxically, the main advantage nanotechnologies grant is precisely multifunctionality: sophisticated therapeutic systems that will be able to detect, navigate towards, and treat complex malignancies at a cellular level. This is only one of the many barriers the field faces when hoping to advance medical technologies from the research bench to the patient's bedside. It should be noted here that this 'energy barrier' may be the reason why many great technological platforms that were developed in academia remain unrealized clinically. Research-

ers (especially young ones) fear that investing time at promoting technologies developed in their lab may come at the expense of academic promotion. At the interest of better treatment, this 'technological death gap' must be bridged. Researchers must be encouraged to take an additional step and make potential new technologies widely available.

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