Expert Opinion

Antibiological barrier nanovector technology for cancer applications

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The advent of sophisticated drug delivery strategies for cancer applications has inundated the scientific and clinical community with new tactics and approaches such as molecular targeting, nanotechnology-based methods and personalised therapies. Unfortunately, the clinical impact has been moderate at best, falling significantly short from revolutionising existing chemotherapeutic methodologies. To this day, a cancer patient has a higher probability of receiving traditional systemically administered drugs than a more sophisticated targeted or nanotechnology-based therapeutic. This is not a reflection upon the novelty or quality of the technologies, but an indication of opportunity for a new approach that offers the realisation of the full potential of these scientific advances. This approach acknowledges the significance of the numerous biological barriers presented in the human body and their sequential nature. It is then recommended that computational mathematical tools are used to predict which nanovectors, surface modifications, therapeutic agents and penetration enhancers to use for a multi-stage drug delivery strategy. An approach where several stages of micro-/nano-vectors are nested within each other and delivered to overcome specific biological barriers to ultimately release a concentrated dose of a therapeutic payload at the intended lesion site. This novel, multi-stage strategy enables efficient localised delivery of chemotoxic drugs that may lead to significant improvements in therapy efficacy, reduced systemic toxicity and decreased total amount of injected drugs.

Keywords: biobarriers, cancer, chemotherapy, drug delivery, multi-stage particle, nanotechnology, nanovector, personalised medicine, rational design

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1. Introduction

The fundamental basis for the administration of drugs is to achieve a favorable therapeutic outcome for the treatment of a medical condition or disease. The evolution of medicinal therapies can be dated back to chewing willow bark and leaves to relieve symptoms of pain and fever, to the intravenous injection of cancer-killing nanofabricated particles deliberately engineered to deliver a potent drug payload at the site of malignancy. It is a fact that such sophisticated chemotherapeutic strategies exist – a few are even commercially available. If this is true, why do conventional chemotherapies still resemble ancient systemic drug delivery approaches, such as ingesting herbal remedies, more closely than the futuristic targeted cancer-fighting agents? The answer to this question is complex and can be attributed to the challenges the body presents to foreign entities.

Conventional cancer chemotherapeutics gain access to the bloodstream through intravenous administration and are required to penetrate the extravascular space and present at the tumor lesion at an adequate concentration such as to inflict lethal toxicity. Unfortunately, only 1 out of 100,000 molecules of drug successfully reaches

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the intended site, permitting the overwhelming majority of the highly toxic, non-discriminating, systemically dispersed, poison to manifest in a number of side affects associated with cancer chemotherapy. This familiar scenario is quantitated in a Kaposi's sarcoma study that demonstrated the percentage concentration of doxorubicin in Kaposi's sarcoma lesions to be ~ 0.001% [1]. This therapeutic phenomenon appears not to be a tumor-specific challenge and, therefore, applies to most malignancies and tumor types [2-5].

The next logical evolutionary step in drug delivery is to create a mechanism to target therapeutics to cancer lesions. In principle, this can be achieved through a variety of strategies, such as physical, biological or molecular targeting of pathologically relevant sites with a desired chemotherapeutic agent. Such targeting mechanisms are already employed in the clinic and are commercially available: liposomal formulations of doxorubicin that exploit leakiness of tumor vasculature [6-8], monoclonal antibody therapy, trastuzumab, that targets the Her-2/neu protein receptor on overexpressing breast cancer cells [9-11], and the small molecule, imatinib, that targets Bcr-Abl, a specific oncoprotein that causes chronic myeloid leukemia [12-14]. However, no level of targeting sophistication will produce substantial benefits in therapeutic index, unless the agents of the therapeutic actions can reach the intended lesion sites at the right dose, which explains why existing treatment strategies have not been totally revolutionized by the advent of the present generation of targeted therapy.

Intrinsic to the body's defense systems are several extremely effective obstacles (collectively termed 'biobarriers') that largely prevent injected chemicals, biomolecules, nanoparticles and any other foreign agents of therapeutic action from reaching their intended destinations. Some of the most notable challenges include physiological barriers (i.e., the reticulo-endothelial system, epithelial/endothelial membranes, cellular drug extrusion mechanisms) and biophysical barriers (i.e., interstitial pressure gradients, transport across extracellular matrix, expression and density of specific tumor receptors). Biobarriers are sequential in nature, and, therefore, the probability of reaching the therapeutic objective is the product of the individual probabilities of overcoming each one of them [15]. A corollary is that any efficient delivery method must be provided with tools that allow it to overcome all of these barriers [15,16]. The requirement of a therapeutic agent to have the components that allow it to cross all barriers and still be small enough for safe vascular injection is a challenge that faces nanotechnology [16]. Injected, nano-scale drug delivery systems, or 'nanovectors', are the ideal candidates to the time-honoured problem of optimising the therapeutic index for treatment (i.e., to maximise efficacy while reducing health-adverse side effects) [15,16].

2. Defining the crucial pathways for nanovectors

The fundamental breakthrough opportunities for nanovector delivery are summarized in three, closely interrelated main aspects: i) the specific recognition of target cells and tissues; ii) the ability to reach the diseased sites where the target cells and tissues are located; and iii), the ability to deliver multiple therapeutic agents and contrast agents for multi-modal imaging. The first two aspects comprise the notion of achieving a preferred, substantially higher concentration of therapeutic action at lesion sites, a phenomenon that will be called 'localization' in this article, as opposed to the term 'targeting' that is often used to identify drugs that provide specific action against a target biological pathway. Each of the three fundamental aspects further articulates into several statements of challenge, and is accordingly presented in what follows.

2.1 Recognising intended cells and tissues

The most widely investigated modalities for the recognition of the cells and tissues against which the therapy is directed involve the attachment of therapeutic agents (in nanoparticle format or not) to biological recognition moieties, such as antibodies, aptamers, and ligands. Although successful in some instances, this approach presents limitations on at least three fronts: i) the specificity of the recognition molecule; ii) the relative local overexpression of the conjugate target molecule receptors, for instance integrins on neovascular endothelium; and iii) the biological recognition of the targeting molecules by the natural defenses of the body. Furthermore, therapeutic antibodies by themselves reach their intended targets only in the ratios of 1 in 10,000 to 1 in 100,000 [17]. Although the improvement of biological targeting is certainly an important direction to pursue, and is being actively investigated by the nanotechnology community [18-23], the use of nanovector delivery affords substantial advantages for what pertains to a broader consideration of target selectivity. These are obtained from the decoupling of the therapeutic action *per se* from the localisation requirements – the latter are left to the nanovector, as opposed to the drug they carry.

The key strategic direction is, thus, the use of multimodal localisation strategies, of which biological affinity-based strategies are but one, on the basis that localisation probabilities are essentially additive: as long as they are non-interfering, each contributes favorably to the achievement of preferred spatial concentrations. Some promising non-biomolecular targeting mechanisms are briefly discussed next.

2.2 Localisation by size and shape

Years of liposome research and clinical use have demonstrated that tailoring nanovector size to match the fenestrations of the cancer neovasculature yields preferential concentration at tumor sites – a phenomenon termed enhanced permeation and retention. Similar considerations apply to the near totality of nanovectors presented in the literature, as they are all spherical or nearly spherical. However, recent advances in nanofabrication technology may open the way towards the development of alternate geometries for injectable, silicon- and polymer-based delivery systems that may be more

effective in concentrating within the diseased microvasculature and tumor mass.

2.3 Localisation by physical properties

The recognition that the surface charge of nanovectors influences tumor uptake is not very recent [24,25], but has been essentially forgotten by the community. Contemporary mathematical models have advanced the understanding of the relationships between surface charge and stromal accumulation [26], and may induce the development of novel classes of nanovectors [26,27].

2.4 Localisation by remote or environmental activation

Regardless of the details of the distribution of injected nanovectors in the body, exquisite localisation of the effect may be attained if the cytotoxic action is released only at the intended target sites, by irradiation with an exogenous, and locally focused source of energy. Remote activation approaches that have been demonstrated in the literature include the triggering of gold nanoshells by near-infrared radiation [28], leading to localized thermal ablation of tumor xenografts in animal models [29-31]. West and colleagues achieved mean tumor temperatures (~ 50ºC) that were significantly higher ($p < 0.001$) than their control mice using near-infrared-radiation-activated gold nanoshells [31]. Literature pertaining to near-infrared radiation thermal ablation approaches for cancer treatment demonstrates that it takes only 8 min at 46°C to kill malignant cells, and 51°C can be lethal after only 2 min [32]. Enhanced photodynamic therapy by targeted silica nanoparticles [33,34], neutron-capture therapy with gadolinium nanoparticles [35], ultrasound-based delivery from lipid-encapsulated microbubbles [36] and the magnetic field activation of cytolysis [37] have also been reported. Localised release may also be activated by environmental conditions, such as metabolic markers [38], and the acidity levels that accompany inflammatory states, infections and neoplastic processes [39]. Therapeutic targeting may be attained by two-step activation methodologies, such as polymer-directed enzyme prodrug therapy and polymer–enzyme liposome therapy [40].

2.5 Endothelial and epithelial barriers

Although circulating nanovectors could be used to target other circulating cells, the majority of potential applications imply a need to leave the circulation and penetrate surrounding tissue. Thus, the first barrier encountered by the vectors is the endothelial cell layer lining blood vessels. The structure of normal endothelium has been extensively described, and an excellent recent review summarizes the present state of knowledge [41]. During the disease progression, the expression of surface receptors can be significantly altered. This is evident, based on the presence of specific endothelial markers that are predominantly expressed on the surface of tumor-associated vascular endothelium, such as integrins, VEGFR2, Delta4 and TEM1 [42]. These significant differences in the expression of surface receptor proteins between normal and tumor endothelium make the angiogenic vessels adjacent to tumors a very attractive target for drug delivery and provide an excellent rationale for vascular targeting and therapeutic exploitation. Therefore, molecules that specifically interact with surface receptors, such as ligands [43], antibodies (VEGFR2 antibody) [44], aptamers [45] and synthetic peptides targeting to integrins (arginine-glycine-aspartic acid [RGD]) [46], could be a potential use for tumor-associated vasculature specific targeting.

Furthermore, the integrin and selectin receptors present on endothelial cells have been extensively studied in the context of blood cell-adhesion, rolling and extravasation. Excellent reviews describe this for erythrocytes [47], leukocytes [48], platelets [49], and > 1800 articles appear in Medline dealing with various aspects of this process. The mechanisms by which circulatory cells leave the vasculature and enter sites of local inflammation and infection have been studied in detail. It has also been proposed that mimicking this behavior (i.e., designing drug carrier particles that carry similar ligands and target the same receptors) may enhance their delivery to infection and inflammation sites [50].

One of the most formidable obstacles to penetration by therapeutic agents has been the blood–brain barrier. However, iron-oxide nanoparticles, by way of their physical properties, have demonstrated considerable efficacy in reaching brain tumors [51-53], and serve locally as signal enhancers for the pre-post and intra-operative mapping of cancer lesions by magnetic resonance and optical imaging means [54-56]. Therefore, these particles are promising candidates as vectors for combined therapeutics and diagnostics.

An example of nanotechnologies for overcoming epithelial/endothelial barriers involves the co-localised delivery of a therapeutic biomolecule with a penetration enhancer, such as a zonula occludens toxin. This acts to open intracellular tight junctions in a short-term, reversible and localised fashion, thus affording transport of the therapeutic biomolecule into the vascular compartment, without concurrent increased risks of opportunistic infection [57].

2.6 Sequestration by the reticulo-endothelial system

The reticulo-endothelial system (RES) is very proficient in clearing small foreign entities in the bloodstream. Liposomal drug delivery technologies have been the most successful and clinically prevalent nanovector in existence, and, therefore, the evolution of the liposome offers much insight into how nanovectors can be engineered to overcome specific biobarriers. The circulatory clearance half-time of liposomes [21,58] was increased from minutes to hours or days, by the attachment of PEG to their surfaces. PEG provides a shielding STEALTH® (Alza, Corp.) effect, delaying recognition and sequestration by the resident macrophages of the RES. Although long-circulation of liposomes by the STEALTH principle is taken for granted today, this provides essentially uncontrolled extension of the circulation time. Unfortunately, the steric stabilization reduces biomolecular targeting capabilities because the PEG

molecules hide antibodies conjugated on the liposomal surfaces. Zalipsky has demonstrated this property in two distinct applications. First, by varying the chain length at which the ligands are anchored, their exposure to the circulatory opsonins and, therefore, their clearance by the RES can be controlled. Thus, folate anchored on ^{2000}PEG (presented as a STEALTH liposome with ^{2000}PEG coating) displayed accelerated clearance compared with both STEALTH liposomes with no folate, and liposomes with a longer (^{3500}PEG) STEALTH layer [59]. However, folate on ^{2000}PEG did not bind as effectively to its receptor as free folate when the STEALTH layer was itself 2 kDa or longer. On the other hand, when the STEALTH layer was shorter than the folate anchor, the binding efficacy was restored. As a core technology that addresses the need to mask and unmask ligands tethered to STEALTH liposomes, Zalipsky has proposed and demonstrated several 'cleavable PEG' concepts, whereby a PEG chain can be cleaved off its lipid anchor by scission of the linker. As the scission should ideally be triggered by a safe level of trigger molecule, and yet not be triggered at native levels of the same molecule, Zalipsky developed cysteine-cleavable disulfides that do not cleave at native (10 μM) cysteine levels but easily cleave at 50 μM cysteine in circulation – a level that is still far lower than the level at which toxicity manifests.

2.7 Interstitial transport

After the endothelium is penetrated, nanoparticles will present in the cellular interstitium, where they will need to move to the surface of target cells. Alternatively, the nanovectors could disintegrate or otherwise leak their contents, which could then transport to their destinations separately. Transport in the interstitium is a complicated process, coupling diffusion, convection and active transport mechanisms. However, both the diffusion and convection of nanoparticles are further complicated by the fact that void spaces in the interstitium are often not much larger than the particles themselves, and the usual transport equations rarely hold. Furthermore, as cancer lesions grow, they develop an increased internal hydrostatic pressure that counters convective extravasation from the vascular compartment into the tumor. This leaves diffusion as the only mechanism of transport of therapeutic agents into the tumor – a very unlikely route for large molecules or nanovectors. This is a very central problem, which unfortunately has not been convincingly addressed in the literature so far.

2.8 Cell internalisation

Transport of nanovectors (or their released contents) through the interstitium will result in their presenting at cell surfaces, both target cells and off-target cells. The next barrier to be overcome would, therefore, be that which is presented by the cell membrane. Both nonspecific binding and surface receptor binding events could trigger further receptor recruitment and surface migration events, to possibly strengthen the binding. Similar transmembrane signaling events could trigger endocytosis of the bound species, resulting in the formation of a vacuole containing the nanoparticle. The particle could release its contents within the vacuole, where degradation of the payload could occur. However, receptor-mediated binding and internalisation processes are affected by the normal function of the receptors, as demonstrated by Annapragada and colleagues [60]. Thus, targeting liposomes via the folate receptor is dependent on the number of folate ligands per individual particle, with an optimum being observed ~ 400/particle. This is attributed to a downregulation of folate receptor recycling following the endocytosis of sufficient folate to satisfy a cell's folate needs [61]. A further complication of receptor targeting is that unique receptors that are only expressed on target tissue are difficult to identify. Thus, in an effort to increase the specificity of receptor targeting, multiple receptor sets constituting a 'fingerprint' of the cell surface must be sought, and nanoparticles tailored to this fingerprint. An example of this approach, showing enhancements in specificity and cell kill has been recently published [62].

2.9 Nuclear or organelle localisation

The ultimate site of action of the drug is likely to be an organelle or intracellular structure where the target is located. Thus, cisplatin (a DNA intercalation compound) is likely to be the most active upon intranuclear localisation. Many of the same considerations as interstitial transport apply here as well: transport through crowded spaces, and likely receptor binding to organelle surfaces followed by an internalization process. Relatively little is known about the processes of organelle localisation and internalization; one of the few well-studied examples is the folate ligand, whereby liposomes targeted with the folate ligand exhibit enhanced nuclear delivery of their payload [63].

3. Delivering multiple agents of therapy

Contemporary cancer therapeutics generally involve the simultaneous use of multiple drugs, based on the need to intervene on a spectrum of cancer-overactive mechanisms possibly acting in a parallel or redundant fashion. The probability of two different drugs reaching the same site at the same time is obtained by multiplying the individual probabilities. In an oversimplified computational example, two drugs with a probability of 1 in 1000 of reaching the intended target only combine to act with a probability of 1 in 1,000,000. At the same time, essentially twice the amount of active agent reaches unintended sites than it would with either one of the agents alone, thus causing much greater, undesired collateral effects. This argues in favor of co-localising multi-drug therapies by incorporation in nanovector carriers.

4. *In silico* rational design and combinatorial methods of vectors synthesis and screening

The pharmaceutical and biotechnological industries have based their successes on the ability to: i) generate large combinatorial

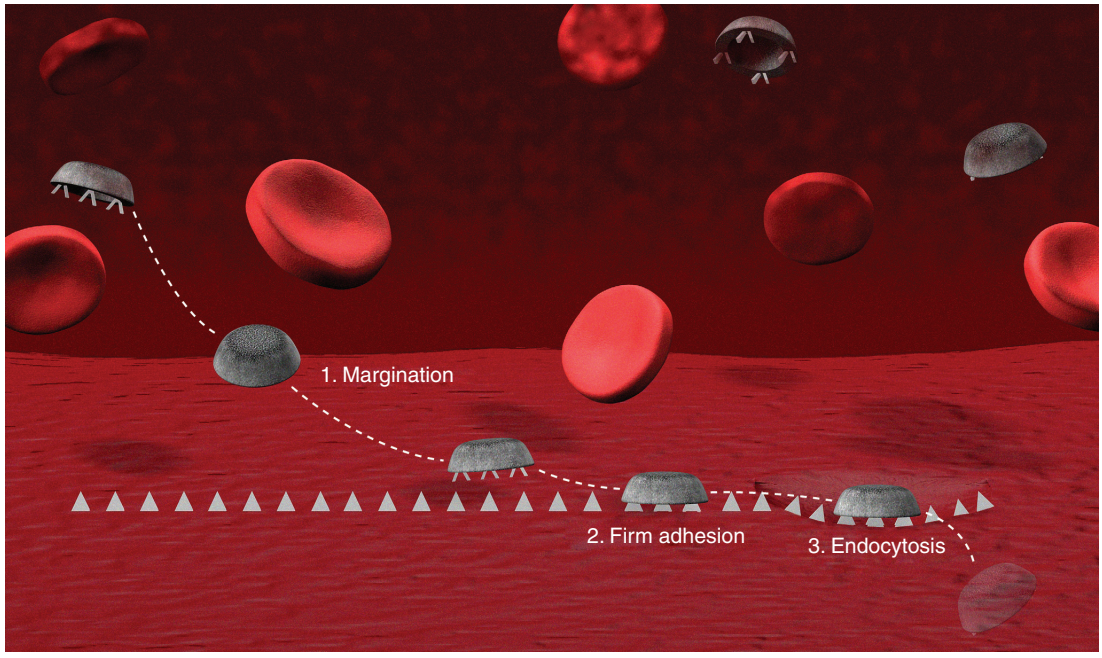

Figure 1. Margination, adhesion and endocytosis: three events in the interaction of a nanovector with a cell layer under a linear shear flow.

libraries of candidate drugs; ii) use high-throughput screening methods to yield a manageable number of 'leads'; and iii) preclinically test them for safety and efficacy. At this time, there are few counterparts to i) and ii) in nanotechnology, and iii) is slowly maturing. A large variety of vectors have been proposed and are being developed with different geometrical, physical and chemical properties that could be used as first-stage and second-stage vectors. They differ in i) size, from few tens of nonometers [64] up to few microns [65]; ii) in shape, from the classical sphere to conical and discoidal [66], oblate spheroidal [67] and elongated as for the nanotubes [68]; iii) in composition, as polymeric [40], metallic [69], silica-based [65] and biological [70]; and in iv) surface-recognising moieties, as antibodies or fragments of antibodies [71], peptides [72] or aptamers [73,74]. With such a large variety, a question arises almost spontaneously: is there any combination of properties such as size, shape, composition and surface physicochemical properties that can identify an optimal nanovector for targeting specifically the diseased microvasculature and the diseased cells within the extravascular space?

Inherent to the design of a vector navigating within the microvasculature seeking diseased endothelial cells, three issues are of importance: i) the margination dynamics, ii) the strength of adhesion, and iii) the control of cell uptake (**Figure 1**).

The term margination dynamics is used here to refer to the lateral drifting of the nanovectors towards the wall of the blood vessels: the endothelium. This feature is of importance because it allows the vector to move in close proximity of the blood vessels, possibly within the 'cell-free layer' [75], and, thus, favoring the search for the diseased vasculature and eventually allowing for firm adhesion, if the conditions are met. Evidently, a nanovector moving, as red blood cells do, in the centre of the bloodstream would only occasionally interact with the vascular walls. The margination of a nanovector can be induced through external force fields, as those due to gravitational, electrical and magnetic forces and particle exclusion in dense flows that attract the vector towards the blood vessel walls. Therefore, margination can be controlled by choosing the shape and the inertia of the nanovector such to to facilitate lateral drifting within the flow [76].

In particular, margination time can be defined as the characteristic time needed by navigating nanovectors to approach and contact the vessel walls. This is an important parameter to judge the utility of a given particle in a particular application: particles with a short margination time are more likely to contact the endothelial wall and arrest firmly on it. Such particles are candidates for use in drug delivery systems. Particles with a long margination time are more likely to circulate in the bloodstream with fewer interactions with the endothelium.

The term strength of adhesion is used here to refer to the ability of a vector to attach firmly at the blood vessels, withstanding the hemodynamic forces. This feature is of importance in that the nanovector should firmly adhere to the diseased endothelial walls to allow for its detection by an external imaging system or to release smaller nanoparticles towards the diseased extravascular space. The strength of adhesion is regulated by specific interactions (ligand–receptor bonds) and

by nonspecific interactions (short-range attractive/repulsive forces) arising at the cell–vector interface. It can be controlled by changing the geometrical (size and shape), biophysical (density and type of the ligand) and surface physicochemical propertiesof the vector.

The formation and disruption of specific molecular bonds at the cell–vector interface is stochastic in nature and has been successfully described through a probabilistic kinetic formulation [77]. In particular, the probability of adhesion (*Pa*) can be defined [78] as the probability of having at least one close ligand–receptor bond through which the hydrodynamic forces are transmitted to the cell membrane. This probability of adhesion can be directly related to the strength of adhesion: the larger the value for *Pa*, the larger the adhesive strength of the particle to the cellular substrate. *Pa* is affected by the geometric features of the vector, such as its size and shape; by the biophysical properties of the cell–vector system, such as the surface physicochemical properties; by the density of the ligand molecule and its type (ligand–receptor affinity); and by physiological parameters such as the shear stress at the wall (μS) and the receptor molecule density (m_r). It has been shown [78] that under the reasonable hypothesis of a small number of ligand–receptor bonds, and a uniform distribution of receptors on the endothelial surface, an optimal radius (R_{adh}) for the firm adhesion of spherical vectors can be derived as a function of the physiological ratio ($\mu S/m_r$) and is given by:

$$R_{adh} = \left(\frac{3}{4\pi}\alpha\right)^{1/3}\left(\frac{m_r}{\mu S}\right)^{\beta} \text{ in } \mu m \quad (1)$$

with $\beta = 0.716$ and α depending on the ligand–receptor family and the physicochemical properties of the vector–cell system. The scaling law introduced with the equation (1) is in good agreement with the experimental results presented in [71], where functionalised polystyrene microspheres with different diameters (5, 10, 15 and 20 μm) were perfused in a laminar flow chamber to derive $R_{adh} \propto (m_r/\mu S)^{\beta}$ with $\beta \cong 0.75$.

It has also been shown [78] that non-spherical particles with an oblate shape should be preferred to spherical vectors, to increase the effective payload for a given probability of adhesion. Oblate spheroidal vectors, compared with classical spherical particles, have a larger interacting surface, which increases the number of specific bonds that can be formed, and a smaller cross-section, which generates smaller hydrodynamic forces. This all explains the larger adhesive strength of spheroidal particles, or their larger size compared with classical spherical particles for a fixed adhesive strength. In capillaries with a wall shear stress ranging between 1 – 10 Pa and a surface receptors density of 10^{14} m^{-2}, the optimal volume for a spherical particle ($\gamma = 1$) would be ranging between ~ 6.3×10^{-2} and 4.4×10^{-4} μm^3, corresponding to a diameter ranging ~ 100 – 500 nm. Liposomal and polymeric spherical particles presently used as drug carriers have a diameter ranging between a few tens of nonometers up to 100 – 200 nm. This characteristic size is sufficiently small to allow passive extravasation across the leaky endothelial walls of a tumor microvasculature (enhanced permeation and retention) [79,80], and it is also within the optimal range for specific adhesion of the particle to endothelial walls. However, under the same physiological conditions, an oblate spheroidal particle with $\gamma = 2$, with the same probability of adhesion as that of a spherical particle with a diameter of 500 nm, would have a volume $V \approx 3.5$ μm^3. This means it has a carrying capacity that is ~ 50-times larger than a classical spherical vector with the same adhesive strength, and, thus, can allow a larger amount of drugs to be released, or a higher intensity contrast-enhancing effect can be achieved at the targeted surface. However, the circulation time and clearance behavior of these oblate particles is unknown, and needs to be investigated.

Finally, the term cell uptake control is used here to refer to the ability of an adherent nanovector to resist cellular uptake by receptor-mediated endocytosis. This feature is of importance in that a nanovector could not release its payload from the interior of an endothelial cell, unless it is the intended target. The endocytic performance of a nanovector can be modeled, as with the strength of adhesion, through manipulation of its geometrical and biophysical features [26]. Recently, Decuzzi and Ferrari [26] have proposed a general formulation to predict the endocytic performances of spherical nanometer particles where the threshold particle radius (R_{th}), the optimal particle radius (R_{opt}) and the characteristic time (τ_w) for endocytosis are estimated as a function of specific and nonspecific particle–cell interactions. In particular, an explicit expression for the minimum threshold radius below which no endocytosis can occur has been presented. It has been demonstrated that the contribution of the nonspecific interactions (e.g., van der Waals forces), is as important as the contribution of the specific interactions related to the formation of molecular bonds at the cell–particle interface.

5. Conclusion

The development of a highly efficient cancer therapeutic has yet to be identified; however, the challenges that relegate present chemotherapeutic strategies to the administration of systemic and nonspecific delivery of toxins are being elucidated and classified as biological barriers. The identification of the most challenging biobarrier that demonstrates the lowest probability of being overcome is truly academic, but regardless of this, a drug delivery strategy that does not permit the crossing of all barriers is a flawed one. Furthermore, the level of challenge presented by a particular barrier is dependent upon the characteristics of the particle to be delivered. For example, a bare liposome carrying a payload of drugs may readily pass the cell wall; however, if injected intravenously, the RES will clear the vectors in minutes. The scientific community is responding to the notion of the existence of sequential biobarriers, as evidenced by literature demonstrating a transition of focus away from targeting the actual tumor to investigating how to localise vectors to the tumor vasculature. Doxil® (Alza) is the

perfect example of a nanovector that has been engineered to avoid a specific biobarrier – in this case using PEG STEALTH liposomes to avoid sequestration by the RES, which enables them to reach the tumor vasculature. The authors of the present review propose to use an *in silico* rational design approach to develop porous silicon micron-sized carrier particles that maximise the probability of multi-stage particles manoeuvring through the vasculature, marginating, recognising and adhering to the tumor vasculature, and delivering their payload of nanovectors. Particle size, shape, charge and surface modifications represent potential multi-stage carrier particle properties that can be modified for the efficient delivery of nanovector payloads at the site of disease.

6. Expert opinion

The ideal injected chemotherapeutic agent would be capable of travelling through the vasculature after intravenous administration, reaching the desired target at full concentration and then selectively killing diseased cells/tissue without any harmful or undesirable side effects. Unfortunately, there is an enormous disparity between the conventional drug delivery strategies and the ideal drugs of the future. It will require a dramatic deviation from conventional methodologies to consider and acknowledge that biobarriers are sequential, and, therefore, that future strategies to overcome them must also be. As an analogy, the Apollo 11 crew did not land on the moon solely by piloting the lunar module to their destination. It took the 3 stages of Saturn V and the command module to escape the earth's atmosphere, navigate the correct lunar trajectory, land on the moon and return back to earth safely. A multi-stage approach to killing cancer integrates several nanovector technologies, biological barrier evading mechanisms and targeting strategies, in addition to a payload of cytotoxic agent(s).

There are at least two significant paradigm shifts that are envisioned to arise from the multiple-stage nanovector philosophy: i) the decoupling of therapeutic action from biological recognition which is typical of nanovectors and ii) the development of new 'personalized' therapies that exploit differences in pathophysiologic signatures through mathematical modeling. Personalised medicine of the future, as defined by the multi-stage approach, will rely upon imaging modalities to offer pathophysiologically relevant patient information regarding numerous physical features (i.e., vascular diameter and tortuosity, tumor vascular fenestration size, blood flow dynamics). The analysis of these physical parameters will suggest the appropriate multi-sage nanovector strategy to employ.

Literature searches offer an overwhelming number of distinct particle variations when considering particle size, shape, charge, surface modification and drug payload/therapeutic effect. The issue is not the lack of novel technology but the need to find an efficient and efficacious methodology to select a reasonable number of vectors for consideration. The proposed rational design approach, based upon computational mathematics, could provide nanovector options for each sequential biological barrier. Within the decade, pharmaceutical companies may employ mathematics to initiate the design of a multi-stage delivery strategy that uses a carrier or 'mothership' particle that is capable of releasing different stages of particles that are nested within one another and designed to circumvent different biobarriers and/or targeted release functions. A first, embodiment of the strategy, designed for intravascular injection, may be conceptualised as follows: stage one vectors are flexible, submicrometer prolate spheroids or disks. Their size and even more importantly, their shape are such that they will travel trough tumor capillaries in the closest possible contact to the endothelium, in the target cancer lesion vasculature, and therefore have the greatest probability of preferentially recognising neo-vascular markers, or penetrating through fenestrations. They have different recognition moieties on their surface, including biologicals (aptamers, antibodies), and will feature detection and optimized adhesion strategies based on physical properties and charge distribution. Upon lodging in the tumor vasculature they release, preferentially in the desired direction (i.e., away from the vasculature), both penetration enhancers and nanoparticles that comprise stage two.

The penetration enhancers can be metalloproteases directed against the basement membrane (such as collagenase IV, MMP-2, MMP-9) and/or toxins against the tight-junction proteins, which are themselves released in multiple timed waves to interfere with different proteins in the tight junction protein sequence. The stage two nanoparticles, spherical and with dimensions of 5 – 30 nm, penetrate the cancer lesion through the fenestrations generated by the permeation enhancers, and selectively direct their cytotoxic payload against tumor or stromal cells that carry surface marker antigens that are conjugated to the single-chain antibodies immobilized on the surface of the nanoparticles. In yet another variant, the stage two nanoparticles are of lipid nature, and fuse with cell membranes. Once in the cytoplasm, they release stage three nanoparticles, which are small enough to cross the nuclear membrane, (i.e., are 3 – 10 nm), and, therefore, can act as delivery agents for nucleic acids or gene therapy. An illustration of the strategy is presented in **Figure 2**, with specific reference to small interfering RNA. Stage two particles of for intravascular or pulmonary delivery could be capable of activation by exogenous energy, such as deep penetrating near-infrared light radiation. Thus, they could lodge deeply in tissue prior to the photodynamic activation of their therapeutic action. A very large number of possible embodiments for multi-stage nanovectors could easily be envisioned, and tailored to the biology of the biobarriers of interest, and through this, to the specific clinical applications.

The future of drug delivery will be based upon a strategy that decouples the present therapeutic dogma most efficiently: to employ the drug molecules for their therapeutic action only, and deliver them to the intended site by vectors that can be preferentially concentrated at desired body locations through

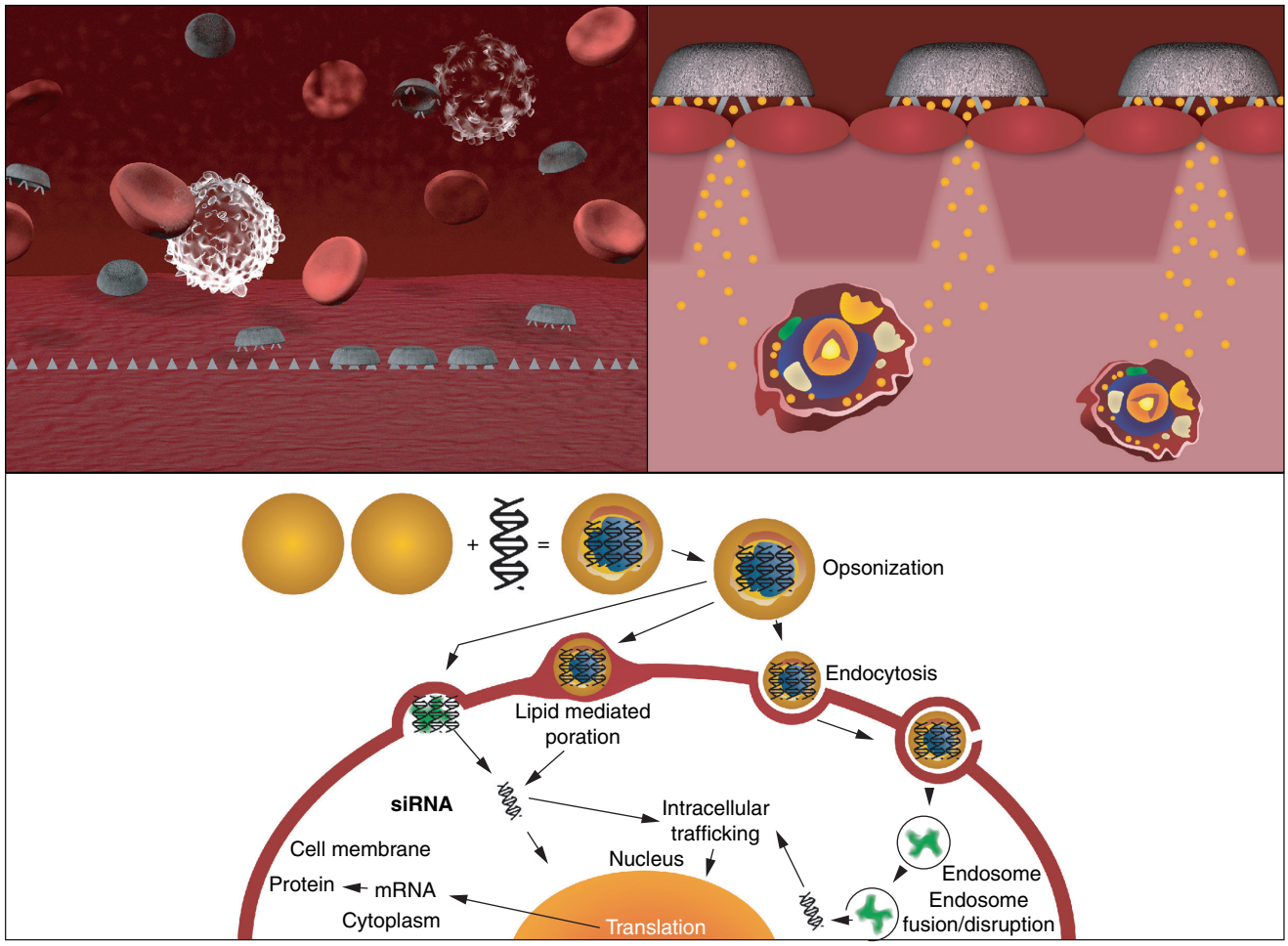

Figure 2. Top-left: rationally designed stage one particles marginate to the vessel wall and adhere to the endothelium. Top-right: stage one particles release a penetration enhancer to break down tight junctions and the basement membrane and release stage two particles – in this instance, liposomes. Bottom: the stage two liposomes interact with the target cell membrane, and then deliver the intended payload – in this example, siRNA.
SiRNA: Small interfering RNA.

the concurrent action of multiple targeting mechanisms. The adoption of such a divergent concept would require the complete overhaul of the fundamental approaches of the pharmaceutical industry: the drive toward the identification of biologically targeted drugs could be replaced by the use of any, nonspecific cytotoxic agent encapsulated within a site-directed, multi-stage nanovector system. Furthermore, a new approach to personalised medicine will also be critical, allowing the pharmaceutical industry to supply a combinatory library of nanoparticle technologies and offer computational rational design tools to prescribe an appropriate treatment without the burden and logistical challenge of synthesizing a personalised drug that may only be applicable for a small number of people. The FDA regulatory approval process is expensive and, thus, remains a formidable deterrent for pharmaceutical companies to develop non-blockbuster drugs. However, large pharmaceutical companies may favorably receive the concept of commercialising an arsenal of stage one nanovector-carrying particle variants for two reasons: i) to allow physicians the ability to choose and load an appropriate FDA-approved stage one particle, based upon their patient's pathophysiology, that can be loaded with a desired nanovector(s) and administered directly to the patient; and ii) to create a vehicle to extend the patent lives of the company's drug portfolio. Realistically, the latter will probably drive the former; however the actual mechanism that enables this new strategy for personalised therapy to become reality is irrelevant as long as new, effective chemotherapeutic methodologies become available to the patient population.

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