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Controlling the morphology of copolymeric vectors for next generation nanomedicine

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Abstract

Amidst the wealth of information that the past few decades of nanomedical research has given us there is one design principle that has emerged as being key for the success of delivery vectors: particle morphology. This review seeks to unpack the various facets of particle morphology that are important for effective integration in vivo. In order to understand the contribution of morphology towards the biophysical function of nanovectors it is important to consider the historical development of such systems and how their physicochemical characteristics are selected. Ultimately, the purpose of this review is to give a clear perspective for the development of future nanovectors and how an integrated approach to their design, with particular focus upon their morphological features (size, shape, stimuli-responsiveness and surface chemistry), is vital for their performance in vitro and in vivo.

Graphical Abstract

Keywords
Nanomedicine, nanoparticles, nanovectors, drug delivery

1. Design principles for nanoparticle-based medicine

The development and implementation in biomedicine of a wide variety of synthetic nanoscale materials reflects the way in which biology recruits a plethora of natural
nanometer-sized structures in order to occupy functional niches [1]. Although the wide array of sub-micron architectures encountered in nature have been fine-tuned over eons of evolutionary development, we can engineer functional nanomaterials that are capable of operating within a biological context by mimicking its design principles [2]. Examples of sub-cellular, nanoscale, objects include mitochondria, exosomes and viruses; all of which have carefully tailored characteristics that enable them to perform certain tasks. Mitochondria and exosomes are crucial for the healthy functioning of cells, whereby metabolism or transport of biological molecules, respectively, is facilitated by distinct characteristics such as size, shape and surface chemistry. Similarly, virus particles have finely tuned structural characteristics in order to evade detection by the immune system and maximise infection to healthy cells. In order to mimic the characteristics of these biological particles, it is necessary to carefully engineer synthetic nanoparticles (NPs), paying attention to all of the physicochemical characteristics of the resulting structure (size, shape, stiffness, temperature/pH/salt behaviour, concentration-dependence, surface chemistry etc.) [3–5]. As we will present in this review, it is of critical importance to adopt an integrated approach when conducting research towards medical applications (such as targeted therapies and diagnostics), which means that the entire process from materials design through to in vitro characterisation and in vivo performance should be seen as one [6]. For example, small changes in the chemical components used in any NP fabrication process (building blocks, solvents, conditions etc.) can have substantial effects upon the resulting structure, which can, in turn, change the in vitro / in vivo performance.

In light of this, it is important that the chemical basis for developing NPs for medical applications (whether therapeutic or diagnostic) should be highly versatile so that control can be achieved at every stage in the fabrication process. Although there are a range of materials that can be used to create nanometric architectures, polymers are the most versatile for this purpose as their chemical composition, length, physical properties and functionality can be easily manipulated to fulfil a given set of design criteria [7]. Biodegradable polymers are especially often applied due to the need for any resulting NP to have minimal toxicity and efficient clearance in vivo [8]. The assembly of in particular block copolymers (BCPs) into well-defined architectures such as spherical micelles, worms, polymersomes and tubes by controlling the balance between hydrophilicity and lipophilicity has been documented and is an excellent basis for the design of NPs with well-defined characteristics [9]. Moreover, utilising the diverse compositions of BCPs allows one to control the size, shape, rigidity, porosity and surface properties of resulting nanostructures, in other words, BCPs permit control over NP morphology; a pre-requisite in the engineering of NPs for medical applications [10].

The justification for placing emphasis on the control of NP morphology is that this has been established as a determining factor leading to biological efficacy [11]. When considering the cellular interactions of NPs, it has been shown that the interplay between particle size, shape, chemical composition and surface properties dictates the rate and pathway of cellular internalization [12]. It has also been demonstrated that the stiffness or rigidity of a particle plays an important role in determining the cellular fate and thereby the
uptake dynamics and, ultimately, the therapeutic efficacy of the system [13]. Not only does the morphology of a particle have a strong effect upon the fate of NPs at the point of interaction with target cells, it also has a significant role when considering behaviour in flow. Understanding this behaviour is essential for their utilisation in medical applications because they will function in a fluidic environment. The justification for such insight is reflected in the non-spherical morphology of erythrocytes, which facilitates their rapid diffusion in the blood stream. It has been reported that high-aspect ratio NPs, in contrast to their spherical counterparts, demonstrate enhanced circulation times and possess favourable uptake properties leading to greater therapeutic efficacy [14]. Other non-spherical architectures have reported favourable properties in vivo, with careful examination of their behaviour highlighting that certain types of particles may have preferences for certain tissues, which could be exploited for site-specific therapeutics [15,16]. Morphology-dependent characteristics of NPs in biological fluids can arise as a consequence of the way in which proteins and other materials interact with (opsonize) particle surfaces in a shape/size dependant fashion [17] and the way in which flow attenuates or enhances the interaction of particles with cell surfaces [18].

Having introduced the idea that morphological control is an essential principle in the engineering of NPs for medical applications, it is pertinent to examine the systems being developed towards this end and the ways in which they are currently being utilised. At present, there are a number of top-down and bottom-up approaches to the generation of morphologically distinct NPs for use in medical applications. Top-down nanofabrication techniques have for example been utilised for particle replication in non-wetting templates (PRINT), which yields polymeric NPs that demonstrate an assortment of morphological characteristics and are easily modified to vary the surface chemistry [19]. Bottom-up engineering of BCP NPs include the self-assembly of amphiphiles into filamentous micelles, which mimic the elongated form of biological structures such as filoviruses [20]. Although having a spherical shape, a lot of attention has been given to the structural characteristics and biofunctionality of BCP vesicles or ‘polymersomes’ due to their ability to encapsulate hydrophilic and/or hydrophobic cargo within a polymeric membrane that can be tuned for permeability, degradability, stimuli-responsiveness and so-called stealth properties [21–23]. The value of stealth behaviour for the circulation of polymeric NPs has been well-documented and is a consequence of the protective layer of hydrophilic polymers on the particle surface [24]. BCP NPs with other morphological characteristics such as discs, compound vesicles and staggered lamellae are also being developed for drug delivery applications and have demonstrated morphology dependant cellular internalization and blood circulation [25].

Pioneering studies into the fabrication and biological testing of such morphologically varied NPs thus promise to greatly expand our capacity to engineer new therapeutic and diagnostic agents that can successfully address medical challenges. However, it is of critical importance to understand the interplay between the chemical, biochemical and biomedical aspects of this research field due to the interdependence between these disciplines. This review will seek to do a number of things: (1) provide a historical context for the
development of NPs towards medical applications, (2) discuss how morphological control is key in the development of next generation nanovectors and (3) present recent findings from the utilisation of morphologically diverse nanovectors in vivo. It is not our intention to supply the reader with a comprehensive discussion on all particles employed in this context and their specific features. Rather, we would like to distil general design criteria and scientific developments in this field from the recent literature to provide the reader with a sense in which direction morphology research in nanomedicine is directed.

2. A concise historical overview on nanomedicine

The motivation behind the development of therapeutic agents, with everything that this implies, is clear. For more than a decade, researchers have been working to increase the efficiency of delivery agents and to progressively develop the complexity of NPs towards certain applications. With so much research having already been conducted in this area is there any space remaining for new approaches or new concepts in this field?

In the development of new drug delivery systems, it is important to reflect upon the historical progression of nanomedicine and to consider the way in which a new approach or methodology, perhaps linked to a new technology, fits into the wider picture. Indeed, advances in nanomedicine reflect an evolutionary process – determined by survival of the fittest. The only way that such an evolutionary process can survive is if there is a strong connection between the chemistry and biology as one provides the basis for structural variations and the other the functional criteria that directs our attention towards certain systems and away from others. However, the evolution of nanomedicine has been a slow process, and there is still much room for improvement and need to deepen our biochemical understanding. Such slow progress may be explained, in the first place, by limited resources and participants, but more recently, development can easily be hindered by a lack of truly inter-disciplinary cooperation whereby chemists might struggle to engage fully with biological considerations and vice versa, which creates a substantial barrier to successful realisation of nanomedical products. With this in mind, it is increasingly appreciated that strong collaboration and cooperation between research fields is the key to success [26].

The concept of nanomedicine was first proposed in the early 20th century by Paul Ehrlich (1854-1915), Robert Koch’s assistant in the Institute for Infectious Diseases, who wasn’t a chemist but a medical scientist. His idea of a ‘Magic Bullet’ [27], able to selectively kill the ‘bad’ and spare the ‘good’, is still an ongoing challenge a hundred years later. Indeed, nowadays, scientists continue to work on a three step strategy for pharmacological applications: transport, targeting and controlled release. But as we know, reaching a successful design takes decades of research, which makes it important to choose the most appropriate design principles and methods to take forward in the future.
Before the 1950s, drugs were mainly delivered orally, by pills or capsules. There were formulations that released the loaded drug immediately upon contact with water without any ability to control the drug release kinetics [28]. Early, macroscopic, drug formulations were intended to release free drugs so that they would reach their designated biological target in an unprotected state. A paradigmatic change occurred thanks to the introduction of new formulations where the properties of polymeric capsules were tailored to achieve control over drug release. By tuning the capsule thickness or composition different delivery profiles were achieved, which facilitated great increase in human health and wellbeing. This technology, born about 60 years ago, started us on the path towards the modern nanoscopic era of medical research. Through the history of nanomedicine there are three evolutionary generations that can be discerned (Fig. 1), with the first starting around 1950 and concerned with overcoming physicochemical barriers arising due to the chemical composition whereas the second generation was concerned with biological barriers – both deepening our understanding and providing solutions. The third, and ongoing, generation of nanomedicine seeks to combine the ability to overcome physicochemical and biological barriers in systems that will be effective for medical purposes [28].

Figure 1. Diagram highlighting the historical development of nanomedicine, as presented in this review, whereby we envisage the present generation of research in this field as an amalgamation of the generations that came before it; building upon knowledge from both the materials and biochemical/biomedical communities.


The first generation of nanomedicines (1950-1980) overcame physicochemical barriers such as poor aqueous solubility or passive diffusion of drug molecules and introduced systems made of large molecular weight materials to counteract these issues. At this early stage, more accessible organs such as the liver and spleen were targeted because foreign entities that entered the body orally were transported to those organs for processing and excretion. The earliest formulations for drug delivery had to come to terms with
physicochemical limitations such as drug dissolution and diffusion and were generally administered orally or transdermally. Indeed, the first polymers that were used for these applications were those that could dissolve in contact with water or the acidic conditions of the stomach. With regards to diffusion of the active agents, it was first necessary to understand the factors that would permit drug-formulations to transfer their contents across the biological lipid barrier. Arising from this was the implementation of liposomes, spherical vesicles comprising a phospholipid bilayer that range in size from 80-300 nm, which can be fabricated by transferring insoluble lipids into aqueous media via an organic solvent. Liposomes have been reported to increase the solubility of drugs and improve their pharmacokinetic properties. Release characteristics of liposomes can be tuned by varying the composition of the membrane so that sensitivity towards pH, osmotic gradient and the surrounding physical environment can be programmed. Examples of marketed liposomes that have shown high efficacy and less toxicity compared to non-liposomal preparations are: liposomal amphotericin B (AmBisome®, Amphotec®, Abelcet®) and liposomal daunorubicin (DaunoXome®) [29]. Other NP formulations, including nanoparticles based upon iron [30] or drug nanocrystals [31], were developed at this time but were not widely utilised due to their unfavourable chemical and functional properties. It emerged at this time that the opsonisation process, by which proteins that exist in biological fluids bind to foreign bodies to facilitate their uptake by phagocytotic cells and eventual excretion, was a significant hurdle for NPs to overcome. To counter this problem, the value of grafting a hydrophilic stealth-like corona onto the surface of a particle in order to create a steric barrier to surface binding was realised – the birth of the concept of PEGylation. Such considerations take us into the second generation of nanomedicines where understanding and overcoming biological barriers is the main focus attention.

2.2. 2nd generation (1980-2010): bio-adaptive systems

The second generation of nanomedicines (1980-2010), was driven by the development of two important technologies for nanotherapeutics; the concept of PEGylation was a design principle that received much attention alongside the ‘Enhanced Permeation and Retention’ (EPR) effect [32]. Such technological developments were critical in overcoming biological barriers that complicate effective targeting and/or drug delivery due to rapid clearance of particles from the blood stream and filtration by the liver and spleen.

The concept of PEGylation, to enhance both the circulation time and the stability (against enzyme attack or immunogenic recognition) of recombinant protein drugs and other particles quickly became a ubiquitous feature of NPs after its introduction in the late 60s [33]. The extension of this concept to sterically-stabilized liposomes, which demonstrated enhanced circulation times and passive targeting properties, started to generate promising results in vivo and resulted in the products such as Doxil® in the mid-90s [34]. Around the same time, drug-loaded BCP micelles, based on synthetic surfactants known as Pluronics® were also developed, with the synthetic BCPs replacing lipids as the basis for the NPs to good effect [35]. Another important discovery was the EPR effect whereby the leaky vasculature of rapidly growing tumours resulted in increased entrapment and retention of
NPs, which can be exploited in the treatment of solid tumours [36]. Although more recent evidence suggests that the EPR effect is only effective in close proximity to leaky vessels, and not throughout a tumour (due perhaps to the low diffusion coefficient of the NPs within the tumour’s extravascular tissues) this remains an important concept in nanomedical research [32]. During the past decade there has been a lot of interest in tumour-targeted drug delivery using NPs to exploit the EPR effect such as protein-based particles with small dimensions that make them suitable for such uptake [37]. However, such NPs operate using passive tumour-targeting where localization is driven by their small size (an important morphological consideration) and not specific recognition of tumour or neovascular targets.

Although this generation of NPs yielded numerous innovative chemical systems and facilitated a deeper understanding into the biochemical principles of nanomedicine, there were significant problems encountered when translating this into clinical trials. A lack of particle specificity and high toxicity meant that discoveries such as PEGylation and the EPR effect were not enough; further design principles needed to be developed. There was need to find new design criteria that would combine favourable physicochemical characteristics, with an ability to integrate within a biological context, which we are constantly deepening our knowledge of, whilst having greater functional specificity and control [28]. Being able to engineer truly biocompatible NPs would allow us to create nanovectors, which could be tailored for different targeting applications without interfering with other biological processes and systems “capable of transporting and delivering one or more bioactive molecules, including therapeutic agents and imaging contrast enhancers” [29]. For the next generation of so-called ‘smart’ nanovectors (NVs) the goal is to achieve specific targeting whilst maintaining favourable control over peripheral behaviour in vivo (circulation time, retention, toxicity etc.) so that clinical trials will be successful and we can generate products that will positively impact human health.

2.3. 3rd generation (work in progress): targeted nanovectors

In order to successfully overcome physicochemical and biological barriers that were previously discussed, whilst introducing targeting capacity into NVs is a challenge that, at the present time, draws together expertise from numerous fields of research from polymer and materials chemistry to biochemistry, cellular biology and clinical medicine. However challenging, the development of NVs for active targeting necessitates the attachment of molecules that will engage in some form of receptor/effector interaction with the target tissue for the purpose of selective activation, identification or immolation, which can be accomplished using specific antigens or molecular motifs such as peptides [32]. This introduces numerous complications such as the need to understand the structure/function properties of appropriate target groups, chemical methods for their attachment to NVs and how, after attachment, is the native biochemistry influenced. Because any ‘smart’ vector needs to integrate into a biochemical ocean of functionality it is important that morphological changes in the size, shape and surface (important for targeting) be carefully characterised and tailored appropriately. Furthermore, the greatest challenge faced in this research might be to avoid undesirable interactions of a targeting NV with any other biological interface before it
can reach its designated target site. With this in mind, it becomes more important than ever that the fundamental design principles be attended to so that the structural basis of NVs is as suitable as possible for in vivo applications, which is where morphological control becomes of great interest.

Another aspect of smart NVs is that they are capable of controlling the release or delivery of their functional cargo. If the goal of the NV is to accomplish interaction with the immune system in order to facilitate, for example, T-cell activation then effective display and accessibility of surface motifs is highly important and these should not become deactivated in biological medium [38]. Alternatively, if drug release or enzyme delivery is the desired NV function then control over the way in which the active agent is released in time, and also in space, is very important. A design concept that is increasingly being exploited in NV design is stimulus-responsiveness, whereby physical responses to a certain stimulus (temperature, pH, osmotic stress, light and chemical triggers such as [O2] or [H2O2]) can be incorporated into the structural components in order to perform the desired function under the desired set of conditions. Polymeric NVs, in particular, are amenable to the incorporation of stimuli responsive components through blending with chemically responsive structural units [39]. Structures that can undergo morphogenic transformation via enzymatic degradation, pH-induced charge reversal, sol-gel transitions, redox sensitive bond cleavage, photo-induced cleavage or isomerization can be utilised to target biological environments that display the requisite parameters (or in the case of photosensitivity introduce an external stimulus) [40–42]. Such changes in the morphological properties of NVs would facilitate a concomitant activation or release of functional cargo in order to address the particular diseased tissue and, as such, have great clinical potential [43]. Another approach to stimuli-responsive selectivity is to programme the release of functional components as a result of changes in the local environment such as redox or pH-induced cleavage of drug-polymer scaffolds [25,44] or pH-induced charge reversal leading to release of siRNA [44].

Most recently, conventional NVs, which are largely based on spherical constructs with fixed nanometric dimensions, are being redesigned in order to gain greater control over the morphology with promising results. Facilitated by advances in materials science and nanofabrication methodologies, the ability to finely tune the geometric parameters of NPs has created new opportunities for NV design [5,45,46]. In the following chapter we will examine the physical and biological factors that contribute towards the recent focus upon controlled morphology as a key design principle in NV development for the next generation of nanomedical technologies.

3. Aspects of morphology for nanovector development

Having presented the current state-of-the-art in terms of the development of biomedical NVs, it is useful to describe the specific morphological features of these systems with respect to the physicochemical and biochemical properties and how these are utilised for medical applications.
3.1. Morphological engineering of polymeric nanostructures

Top-down fabrication is an attractive approach for the production of morphologically discrete NVs (Fig. 2). Two attractive methods exist for this purpose: film stretching and PRINT [47]. By film stretching, polymer-based spherical NPs can be elongated into nanorods through a process of heating, stretching, cooling and template dissolution [48]. This process is capable of generating particles with complex behaviour derived from the polymeric components, such as stimulated shape transformation [49]. The PRINT methodology utilises constant processing where a porous template is used to produce particles of the desired nano-geometry [19,50–52]. The non-adherent templates are produced by casting a chemically-resistant, rigid polymeric mould from a photolithographically patterned silicon wafer. The versatility of such a methodology has been demonstrated by the formation of multiphase nanorods [53], hydrogel-based [54] and stimuli-responsive particles [55] that can be implemented in nanomedical research. In these processes it is the physical characteristics of the respective polymers, which arise from the chemical structure and form of the polymer chains, which facilitate the conformational restructuring of derived particles via heating and applying mechanical control. The ability to produce particles in a scalable process with a very narrow distribution makes these techniques very appealing; however, limitations exist with regard to template formation and in the range of polymers (such as poly(lactic-co-glycolic acid), cross-linked PEG-acrylates and hydrogels comprising bifunctional silyl ethers) that have suitable thermal and physical properties to make them amenable to this type of processing.

Figure 2. An overview of top-down fabrication methodologies to generate nanoparticles with control over the size and shape: (a) Schematic representation of the Particle Replication In Non-wetting Templates (PRINT) process whereby a particle precursor solution (red) is pressed into an empty mould (green) and, after curing, are harvested from the template into solution. The range of particle morphologies that can be fabricated using the PRINT process includes (b) nano-cylinders, (c) rods, (d) discs, (e) cubes, (f) boomerangs and (g) hex-nuts. (h) Schematic representation of the film stretching process whereby a flexible, template film is utilised to induce shape transformations by liquefying, stretching and solidifying the embedded particles. Film stretching can be utilised to produce particles with (i) spherical, (j) tubular, (k) spherical discs and (l) elliptical discs. Panels (a) & (f) reproduced from [52] (copyright John Wiley & Sons, Inc. 2009), panels (b-e) & (g) reproduced from [51] (copyright American Chemical Society 2008), panels (h-l) reproduced from [48] (copyright National Academy Sciences USA).
In contrast to top-down approaches, the bottom-up design of polymeric NVs has been progressed by a deepening knowledge of the physicochemical principles behind self-assembly as opposed to technological advancements in fabrication methodologies [56] (Fig. 3). In terms of polymer self-assembly the objectives of control and replicability are more challenging than when using advanced fabrication techniques, however, it allows us to access a far more diverse morphological landscape where bio-inspiration plays more of a key role. The supramolecular characteristics of self-assembled systems, which can be varied so effectively through molecular design, are a reflection of the way in which biological materials are fabricated through the assembly of molecular building blocks into living systems under the influence of intermolecular forces [57]. In particular, BCP derived NVs possess a membrane, arising from the amphipathic assembly of the polymer chains, which is also a ubiquitous structural characteristic of living systems [58]. The structural diversity arising from BCP self-assembly has been utilised in the formation of numerous complex systems with distinct morphological characteristics such as nanoreactors and artificial organelles [59]. Although morphological features such as rigidity, size and surface charge can be introduced through well-established means and methodologies, the ability to control shape of BCP architectures can be a much more challenging prospect. Utilising changes in the structure of BCPs, and the conditions in which they are assembled, it is possible to controllably direct the formation of spherical or worm-like micelles, vesicles and assorted compound structures [25,60] where fine control of the balance between the hydrophilic and hydrophobic components is of critical importance. Worm-like micellar NVs, so-called ‘filomicelles’ named by analogy with filoviruses, are composed of PEG-polylactide copolymers that are assembled through dissolution in organic solvent and subsequent dispersion in an aqueous solution with evaporation of the volatile organics yielding the desired NVs [20,61]. Stabilization of therapeutic drugs in the hydrophobic membrane can be easily realized utilizing this methodology and surface characteristics such as functionality and the length of the PEG corona can easily be varied in order to influence the biological performance of the NV. Moreover, fragmentation, degradation and rigidity can all be tailored by varying the molecular characteristics of the hydrophobic portion of the BCPs although this is limited by the overall phase behaviour where filomicelles are bounded by micelle and vesicular structures. The ability of such filomicelles to deliver drugs is dependent upon the ability of the BCP framework to uptake and release non-covalently bound molecules, which is dictated by structural compatibility between guest(s) and host, with low efficiencies and poorly controlled release reflecting a lack of affinity with the hydrophobic inner environment and vice versa [61].
Figure 3. Bottom-up design of nanoparticles via block copolymer self-assembly: (a) Schematic outlining the relationship between BCP structure and resulting morphology. In particular, the packing parameter ($P$), which is the ratio between hydrophobic chain volume ($V$) and the product of the hydrophobic chain length ($l$) and the headgroup area ($a$), dictates whether spherical micelles, worm-like filomicelles or polymersomes are formed. (b) Fabrication of BCP architectures is usually accomplished via the direct hydration methodology where a BCP solution (in organic solvent) is gradually diluted by water, which results in BCP desolvation and self-assembly. (c) Phase diagram of poly(ethylene oxide)-block-poly($\varepsilon$-caprolactone/D,L-lactide) BCP self-assembly demonstrating that the morphology of the resulting architecture can be controlled through molecular engineering, which in this instance is the balance between hydrophobic tail mass ($M_{\text{CH}_2}$) and hydrophilic mass fraction ($f_{\text{hydrophilic}}$). Panel (a) reproduced from [10] (copyright John Wiley & Sons, Inc. 2009), panel (b) reproduced from [47] and panel (c) reproduced from [20] (copyright Royal Society of Chemistry 2013 & 2016).

In contrast to filomicelles, where the morphology of the structure is fixed in the initial self-assembly process, inducement of shape transformations can also be very useful in creating different types of NV. Vesicular structures, of the like formed by both lipids and BCPs, can under certain conditions undergo shape transformation in response to physical factors as a result of thermodynamic restructuring of polymer chains and surface topology [62,63]. Inducing shape transformations in polymersomes allows us to extend the versatility of this system towards different morphologies (Fig. 4). Physically, shape transformation can be accomplished through out-of-equilibrium processing of polymersomes that have complementary chemical features (such as rigidity and porosity) so that otherwise inaccessible forms can be accessed through careful control of conditions such as the composition of organic solvents, rate of addition of aqueous solution, temperature cycling, osmotic shock and chemical cross-linking [64,65]. In particular, nanotubes (tubular
polymersomes) are exciting candidates for the development of NVs due to their high aspect ratio and potential for immunotherapy, alongside drug delivery. Nanotubes have been engineered through chemical cross-linking using bio-orthogonal click chemistry [66] or polymer blending in a film rehydration process [67]. Recognising the importance of using biodegradable polymers in the design of materials for medical applications [68] we have recently developed a facile methodology for the generation of biodegradable nanotubes via the osmotically-induced elongation of PEG-polylactide polymersomes [69]. Through tailoring the balance between membrane flexibility, organic solvent composition and ionic gradient it was possible to transform the polymersome membrane, while in a flexible state, into tubes that became fixed once the organic solvent was removed by dialysis; a kind of out-of-equilibrium processing. With this in mind, it is necessary to examine the ways in which these NVs are being utilised for in vitro and in vivo studies in order to better understand the biophysical consequences of their morphological re-engineering.

![Methodologies for the shape transformation of spherical polymersomes into nanotubes using either (a) chemical cross-linking of the inner (hydrophobic) polymer chains or (b) osmotically-induced volume reduction coupled with changing solvent composition in order to lock-up the tubular morphology. Panel (a) reproduced from [66] and panel (b) reproduced from [69].](image)

3.2. Impact of nanovector morphology on performance in vitro and in vivo

To quote the author: “few people care that bacteria have different shapes. Which is a shame, because the bacteria seem to care very much.” [70], which is to say that bacteria are an excellent example of the biophysical advantages of complex morphology. Far from being inconsequential, the diverse morphologies exhibited by bacteria are an essential part of their evolutionary tuning; their form is as important as their genetic and biochemical contents. Specific advantages of the various morphologies exhibited in bacteria include surface-to-volume ratio effects for nutrient transport in cells of varying sizes, external and internal diffusional advantages for elongated cells, film formation or surface attachment for rod-like and filamentous cells and, importantly, both smaller and elongated cells appear to circumvent
cellular predation and clearance from the body [70] – all excellent characteristics to impart to next generation NVs. Moreover, when considering the behaviour of nanoscopic architectures it is imperative to consider the mechanisms by which these would interact with individual cells and, in particular, the plasma membrane. Endocytotic transport of macromolecules and particles across the membrane into the cell can occur via several mechanisms but are widely categorised into phagocytosis, which only occurs in certain cells that are capable of ‘devouring’ their surroundings, or pinocytosis, whereby cells ‘drink’ their environment. Uptake rates can be enhanced and uptake mechanisms controlled through non-specific and (to a greater degree) specific binding to the surface receptors, which are a vital mechanism by which cells interact with their surroundings [71].

The general principles that relate the physicochemical characteristics of particles to cellular endocytosis are well understood, having been the focus of much research for the last 10 years [72]. The size and surface chemistry of particles has been shown to play an important role in cellular internalization due to the interactions with the plasma membrane, which is negatively charged, and the ability of differently-sized particles to affect the energetics of membrane deformation, which precedes uptake [73]. In other studies, it has been demonstrated that excessive particle stiffness can impede cellular uptake [13] and it is possible to add cholesterol, a ubiquitous plasticizer of biological membranes, to a polymersome in order to enhance uptake [74]. Enhanced cellular uptake has also been demonstrated as a consequence of imbuing NVs with non-spherical shapes, which needs to be investigated more extensively in order to give more extensive understanding [25]. Moreover, particle shape has been identified as a critical factor in particle uptake by phagocytes, with non-spherical morphologies giving rise to a range of behaviours [75,76]. It has also been demonstrated that the aspect ratio of rod-like NPs impacts cellular internalization, with different pathways being triggered depending upon the particle length [77]. One striking challenge in this area of research is that different cell types possess unique characteristics and therefore it is very difficult to comprehensively understand the mode of action of particular NVs in disparate cellular environments. Whilst the current state of knowledge gives us excellent indication that we are on the correct trajectory [78], there is great need for collaborative efforts to unpack the consequences of NV morphology upon the diverse range of cellular interactions. Moreover, cellular trafficking of NVs is also of great importance in order to understand the internal processing of particles by the cell. Up to this point most work has focussed upon cellular uptake, and the examples of NVs that can release their cargo into the cytosol or even bypass the endosomal system and wholly enter the cytosol are limited, warranting more research in this area [79]. Selected examples of in vitro studies that have examined the effect of particle morphology upon cellular interactions are presented in figure 5.
Figure 5. The role of NV morphology in dictating the mode of interaction with cells and their endocytosis is under much investigation and is an important basis for understanding how such particles will perform in vivo. In vitro studies have demonstrated that (a) multiple endocytotic pathways are used by bone-derived macrophages when endocytosing 100 nm NPs, (b) both size and morphology can impact the amount of cellular uptake, (c, d) the degree to which particles become either attached, internalized or phagocytosed varies with morphology. Panel (a) reproduced from [80] (copyright Elsevier 2014), panel (b) reproduced from [50] (copyright Royal Society of Chemistry 2006), panel (c) reproduced from [76] and panel (d) reproduced from [75] (copyright National Academy of Sciences USA 2006).

As has been already stated the effect of NV morphology upon its performance in vivo depends upon its ability to permeate various tissues and perform well in the bloodstream, so that it can circulate freely whilst being capable of interacting with its environment and, in particular, vascular tissues. It has been shown from modelling the flow characteristics of elongated particles that they would, in the bloodstream, naturally drift towards the vessel walls and thereby be more suitable for targeting applications due to this intelligent behaviour [18,81,82]. In other work, rod-shaped particles have shown greater transport across intestinal cells compared to their spherical counterparts, both with active targeting (via biotinylation) enhancing their accumulation at the desired cells, laying the foundation for their development towards oral drug delivery applications; however, development of biodegradable NVs that display such morphology is imperative [15]. Despite such favourable in vitro behaviour, this would all be useless if the NV tended to be opsonized and cleared by macrophages, which is where the advantageous surface characteristics of BCPs can synergistically enhance performance by providing stealth properties. Surface bound PEG, a key structural component of BCPs, and similar hydrophilic polymers provide stealth character in evading the phagocytotic system in a process that is not comprehensively understood [24]. A recent study has shown that protein binding to specific surface polymers is an important feature of polymers like PEG, which can preferentially bind certain serum proteins over others, which leads to stealth properties as a secondary effect [83]. If stealth is a secondary effect of protein binding then more research should be conducted to understand the nature of protein
interactions with the hydrophilic corona of NVs as this may improve our ability to control NV performance \textit{in vivo} through morphological tuning.

**Figure 6.** The effect of NV morphology upon circulation times and biodistribution times \textit{in vivo} has been presented in a number of studies. (a) The circulation times of filomicelles in rodent models were shown to be much greater than spherical counterparts (left graph), moreover, increasing the length of filomicelles from 2-8 µm increased the circulation time. (b) Enhanced filomicelle circulation times have been attributed to their reduced internalization by macrophages in flow, compared to that of spherical vesicles. (c) Studies into the shape-dependant cellular association of various particles under dynamic and static conditions highlight that elongated particles also tend to interact more effectively with their surroundings under flow conditions (mimicking the bloodstream). (d) Biodistribution characteristics of various particles highlight that particle morphology not only affects behaviour in the bloodstream but also the localization in various tissues, with cylindrical particles showing the least accumulation in the kidneys and greatest in the kidney. Panels (a) & (b) reproduced from [14] (copyright Nature Publishing Group 2007), panel (c) reproduced from [18] (copyright American Chemical Society 2016) and panel (d) reproduced from [84].

A more detailed understanding of the effect of particle morphology upon biophysical behaviour will aid in engineering NVs and bring us closer to a tangible system that can be used in nanomedicine. Although we have made much progress there is still a great deal of information detailed \textit{in vitro} studies can yield about suitable design criteria for NVs. There are currently a number of NVs being developed further by studying their nanomedical capabilities \textit{in vivo}, an important precursor to clinical trials. However, as we continue this scientific endeavour, there will be new synthesis strategies, materials and test models that are being developed and so the process of NV development must be integrative as we progress in a cyclic rather than linear fashion towards the end goal of medical application.[85]

4. Developing the new generation of nanovectors for medical applications

In the last section of this review it is pertinent to discuss some of the ways in which NV morphology is being utilised to improve \textit{in vivo} performance (Fig. 6). An excellent example of this is the development of artificial (polymeric) erythrocytes that possess a
discoidal shape, mechanical flexibility, biochemically mediated aggregation and heteromultivalent presentation of ligands for platelet binding and wound targeting [86]. Such morphologically diverse NVs show high specific adhesion and limited non-specific interactions with excellent haemostatic capability over more rigid, spherical counterparts, due to their propensity to marginate to the vascular wall, which results in a substantial reduction of bleeding time in vivo. In other work, increase in the circulation time of filomicelles has been related to their elongated form, which performs well in flow and has been shown to prevent phagocytosis through flow-induced shear forces, resulting in improved tumour-shrinkage in mice models [14]. Moreover, filomicelles have been used to increase the maximum tolerated dose of the chemotherapeutic paclitaxel and enhance tumour penetration as a consequence of their flexibility and form [87]. Implementation of such NVs in combination with radiotherapy has also been shown to enhance therapeutic efficacy, which means that such systems can be used alongside existing treatments to improve success [20]. With this in mind, the degree to which morphology impacts biodistribution is of significant interest. It has been shown that, after injection, morphologically diverse particles (spherical, discoidal, cylindrical and hemispherical) have contrasting organ accumulation characteristics, with discs appearing to avoid the liver to the greatest extent [84]; highlighting another way in which morphology determines NV behaviour, and therefore efficacy, in vivo. There have also been a number of studies that have used NVs with in-built stimuli responsiveness that have demonstrated tumour-specific activity through pH dependant release of chemotherapeutic agents, oxidative stress induced release of siRNA and photoinduced cytotoxicity [43]. Overall, the importance of morphology on in vivo performance is clear and should be considered as a fundamental principle in next generation nanomedicine [88].

The final stage in preparing smart NVs for medical applications requires functional modification of the surface in order to facilitate adhesion to the desired site of accumulation. There are a number of methodologies that have been employed to accomplish NV targeting that include the covalent attachment of antibodies, nucleic acid aptamers, peptides, folic acid and carbohydrates. Moreover, ‘smart’ behaviour can also be built-in at this stage in the design of NVs through the generation of cell-targeting motifs that only become active under certain conditions, such as low pH, which further enhances their specificity and therapeutic efficacy [89]. The latter property can well be used to overcome established challenges associated with reductions in circulation time of NVs, caused by increased non-specific binding to undesirable tissues as a result of the presentation of targeting motifs displayed on their surface [54]. An important consequence of morphological tuning, apart from the ability of small-sized NVs to bypass cellular barriers more readily, is the synergistic enhancement of targeting ability of elongated particles. Elongated NVs that possess targeting motifs, such as antibodies, displayed on their surface are capable of engaging in multiple binding events along their length that can more firmly anchor them at the cellular surface and facilitate uptake or vicinal drug delivery [90,91]. However, it should be noted that there are complexities relating to ligand density on the surface of the NV as it has been described that excessive cellular binding can also inhibit uptake [92], which means that this parameter also requires optimisation. Rod-like NVs have shown reduced non-specific and enhanced specific uptake (through antigen display) in breast cancer cell lines when compared to spherical
particles, with greater inhibition of cell growth [93]. It has also been shown that in certain applications the interplay between size and shape is more complex with immune responses of antigen-modified particles varying between spherical and rod-like particles [94], which highlights that morphology should be varied in response to biochemical findings. Further examples of successfully implemented NVs that utilise the favourable properties of shape towards increasing their therapeutic effect are now in development and hopefully, as a result of the present review, will be seen as the figurative descendant of the past few decades of nanomedical research.

The future development and success of nanomedicine is reliant upon the exerted effort of materials science, biochemistry and medical professionals to collaborate and refine our understanding of what makes a good NV. Up to this point, we have deepened our understanding of the effect of size, shape and surface chemistry upon aspects of NV behaviour such as cellular uptake, circulation, targeting and biodistribution but there is a lot more to do. In general, the use of biodegradable components should be a ubiquitous principle due to the need for biocompatibility; however, all of the knowledge we have gained up to this point has been invaluable in developing morphological design principles that can make an effective NV.

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