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Emerging innate biological properties of nano-drug delivery systems: A focus on PAMAM dendrimers and their clinical potential

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ABSTRACT

Drug delivery systems or vectors are usually needed to improve the bioavailability and effectiveness of a drug through improving its pharmacokinetics/pharmacodynamics at an organ, tissue or cellular level. However, emerging technologies with sensitive readouts as well as a greater understanding of physiological/biological systems have revealed that polymeric drug delivery systems are not biologically inert but can have innate or intrinsic biological actions. In this article, we review the emerging multiple innate biological/toxicological properties of naked polyamidoamine (PAMAM) dendrimer delivery systems in the absence of any drug cargo and discuss their correlation with the defined physicochemical properties of PAMAMs in terms of molecular size (generation), architecture, surface charge and chemistry. Further, we assess whether any of the reported intrinsic biological actions of PAMAMs such as their antimicrobial activity or their ability to sequester glucose and modulate key protein interactions or cell signaling pathways, can be exploited clinically such as in the treatment of diabetes and its complications.

Contents

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1. Introduction

The effectiveness of a pharmacological agent (e.g. drug or vaccine) is dependent on its ability to reach the right target organ, tissue or intracellular site of action in an intact or active (right) form for a sufficient (right) period of time to exert the desired (right) biological activity. Drug delivery systems or vectors are usually needed to improve the bioavailability and effectiveness of an active agent through improving its pharmacokinetics at an organ, tissue or cellular level (see also [1,2]. The drug carrier system offers, amongst others, one or more of the following properties: protection of the drug from degradation in the biological milieu [3–6], improving its passage across biological barriers [7–11] overcoming drug efflux transporters [12,13], avoiding or delaying elimination/excretion [14–16], enhancing cell binding, uptake, trafficking and intracellular localization to the desired subcellular compartments [1,6,17–19]. Delivery systems can often be needed for classical small molecule drugs (e.g. poorly water-soluble drugs with low bioavailability [20]; however, they are almost always necessary for macromolecular drugs such as those emerging from the ongoing biotechnological revolution that include the recent clinically-approved nucleic acid-based therapeutics (antisense oligonucleotides, RNA-interference/siRNA [21–23] and mRNA-based COVID-19 vaccines [24]. Further, they will also be required for delivering the more recently developed macromolecular gene editing technologies such as CRISPR/Cas9 (clustered interspaced short palindromic repeats/CRISPR-associated protein 9) that are undergoing human clinical trials evaluation [25–28]. The active agents of these therapeutics are generally biologically unstable, or of high molecular weight and often negatively charged (i.e. polyanionic) – characteristics that, without the use of an appropriate delivery system, would otherwise prevent their passage through the body to their intracellular sites of action [21,23]. Although a lipid-based delivery system (or liposome technology) has been employed for the improved delivery of some recently approved siRNA drugs and mRNA-based COVID-19 vaccines, there are a plethora of drug delivery systems or vectors, including natural and synthetic polymers, peptides and lipids/liposomes, that can be used to improve the delivery of small molecule or macromolecular therapeutic agents. A review of these is beyond the scope of this article and the reader is referred to more pertinent literature on this topic [1,2,21–23,29].

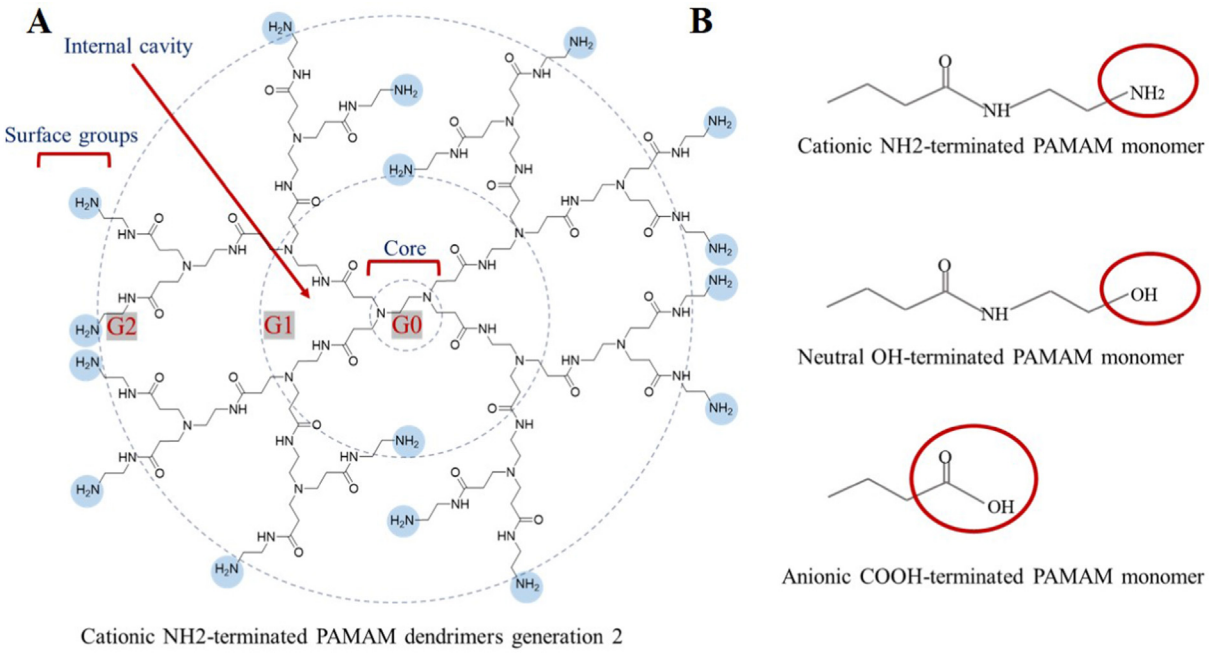
There is a general consensus that drug delivery systems, in addition to improving the delivery of the active agent through modulation of its pharmacokinetics/pharmacodynamics, must be biocompatible and exhibit little or no cellular toxicity [23,30–32]. Traditionally, biocompatible delivery systems, identified through screening of the gross biological properties *in vitro* and *in vivo*, have largely been considered as being "biologically inert". However, with the advent of -omics technologies (e.g. transcriptomics and proteomics) and highly sensitive, high throughput screening platforms, the emerging field of nanotoxicology can now identify subtle, often early, biological effects at the cellular level (genomic/proteomic) that might be predictive of the gross toxicological and biological properties of nanomaterials used in drug delivery systems including interactions of excipients with drug transporters and drug-drug interactions [22,23,31,33–36]. Indeed, we were the first group to show commercially available gene-transfection agents such as lipofectin and oligofectamine as well as linear and branched polyethylene, polypropylenimine and polyamido(amine) (PAMAM) dendrimers induced multiple gene expression changes in human cells (for a review see [22,23,31]. In the context of using these delivery systems in gene silencing studies, where usually only one target-gene change is desired, multiple gene changes induced by the delivery vector would be undesirable as they may mask gene silencing activity and/or contribute to the off-target effects [30,31]. Thus, it was proposed that drug-delivery systems for gene-based therapeutics should also be screened for "geno-compatibility" in toxicogenomic analysis using transcriptomics [22,23,31]. More recently, it has been shown that drug delivery systems including PAMAM dendrimers [37], lipids [38] and cell penetrating agents [32] also modulate protein cell signaling cascades in living systems and thus can exert intrinsic/innate biological actions beyond their effects on drug delivery (see also [39]).

The concept of intrinsically biologically active or bioactive polymers is not novel *per se* but has been known for many years [39,40]. Indeed, several synthetic polymer products have been commercially available for some time as clinically-approved sequestrants of ions or toxins (e.g. sodium polystyrene (Kayexalate®)) and as plasma volume expanders (e.g. Hydroxyethyl starch 130/0.4 (Voluven®) and more recently as immunomodulators (Glatiramer (Copaxone®)) [41–44]. In addition, natural polymers such as nucleic acids, proteins, and carbohydrates have obvious biological activity necessary for life-processes and some are also available as clinically-approved drugs (e.g. Recombinant Insulin is a well-known therapy for reducing blood glucose levels in diabetic patients [45]; and the recent mRNA vaccines are used in SARS-CoV-2/COVID-19 infections [46]. However, synthetic polymers primarily adopted for use as drug delivery systems, were generally not "designed" to have any specific biological action of their own but are now emerging to be intrinsically bioactive often with multiple biological actions.

In this review, we will focus on highlighting the emerging multiple biological/toxicological properties of naked PAMAM dendrimer delivery systems (i.e. in the absence of any drug cargo attached to the polymers). For literature on PAMAM-drug conjugates, the reader is referred to other recent reviews [47–50]. Herein, we further assess whether any of the reported intrinsic biological actions of PAMAMs such as their antimicrobial activity or their ability to sequester glucose and modulate key protein interactions and cell signaling pathways, can be exploited clinically (e.g. in the treatment of diabetes and its complications).

2. Overview of PAMAM dendrimers

Polyamidoamine (PAMAM) or "starburst" dendrimers (see Fig. 1A) were the first complete nano-sized family of dendrimer structures to be chemically synthesized, characterized and commercialized [51] (for review see also [49,50,52,53]. The term "dendrimer" is derived from the Greek words, "dendron" and "meros", meaning "tree" and "parts"and refers to their characteristic branched-like structure. PAMAM dendrimers are generally spheroidal, highly branched, cascade polymers, the size and surface functional group and charge of which can be finely controlled



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Generation	Molecular Weight (Da)	Measured Diameter (nm)	Number of Surface Groups
0	517	1.5	4
1	1,430	2.2	8
2	3,256	2.9	16
3	6,909	3.6	32
4	14,215	4.5	64
5	28,826	5.4	128
6	58,048	6.7	256
7	116,493	8.1	512
8	233,383	9.7	1,024
9	467,162	11.4	2,048
10	934,720	13.5	4,096

Fig. 1. Structural and physicochemical properties of PAMAMs. (A) Schematic showing the chemical structure of G2 amino-terminated PAMAM dendrimer (the core, surface amino groups and internal cavity for drug entrapment or sequestration are highlighted). (B) Structure of cationic, neutral and anionic surface functional groups of PAMAM dendrimers. (C) A table summarising the molecular weight (Daltons), measured diameter (nm), and number of surface groups of different generations of PAMAMs (G0-G10).

during synthesis [52–56]. PAMAM dendrimers can be synthesized by several strategies including divergent step-growth polymerization in a layer-by-layer manner (expressed in 'generations' or "G") typically around an initiator 2-carbon ethylenediamine core unit; though ammonia and cystamine are also commonly used core structures [50]). Thus, layers of radially repeating units attached to the core lead to progressive generations of dendrimers (see Fig. 1A) with defined molecular structure/architecture, branching points, terminal functional chemistry and very low polydispersity [50,52,54]. Each successive generation leads to an increased diameter and molecular weight with twice the number of terminal surface groups as its immediate precursor (see Fig. 1C. In addition to the classical cationic PAMAM with their amino-terminal surface chemistry ((–NH2); hydroxyl- (–OH, neutral) and carboxyl- (–COOH, anionic) surface chemistries (see Fig. 1B) are also commercially available. The cationic and neutral PAMAMs are available as full generation dendrimers (G1 through G10) whereas the anionic PAMAMs are produced in half generations (e.g. G1.5 to G9.5). Thus, PAMAM dendrimers are available as a homologous series of polymer structures with increasing molecular weight (or generations) and different surface chemistries that provide a defined set of physiochemical properties to evaluate structure–activity relationships in drug delivery as well as in the biological actions of these unique nano-structures.

As an additional advantage, post-synthesis engineering of PAMAM dendrimers to produce alternative, often open, structural architectures is also possible. For example, following a controlled solvolytic procedure at high temperature, the so-called "fractured" or "activated" dendrimer architectures can be produced that have reduced internal structural branching and increased internal cavity volume. Such PAMAM nanoarchitectures allow for improved drug entrapment and delivery through increasing the capacity of drug-pay-load that can be physically carried internally within the voids of the dendrimer [57,58]. SuperFect (SF) is an example of a commercially available fractured (activated) generation 6 (G6), cationic (amino-terminated) PAMAM dendrimer.

Thus, PAMAM dendrimers have been considered ideally suited to serve as non-viral drug delivery vectors whereby drug cargo can be carried and protected either within the internal cavities (see Fig. 1A), or bound to the surface groups or even a combination

of the two approaches- a strategy that might be useful for drug combination therapies. Typically, small molecule drugs are those that are entrapped within dendrimer cavities, so as to improve solubility, reduce toxicity and possibly mask taste with the ultimate goal of improving the pharmacokinetics and bioavailability of the active agent (for a recent review see [49]. Entrapment capacity for drugs is greater with activated or lower generation PAMAMs (typically below G4) that have open structures compared to the higher generations (>G6) that exhibit a more rigid surface due to high branching and surface group density [58,59]. Furthermore, drugs or active agents can also be attached to the dendrimer surface through electrostatic adsorption or covalent conjugation (e.g. for recent reviews see [47,49]).

Cationic amino-terminated PAMAM dendrimers, because of their positive charge and ability to bind to negatively charged nucleic acids as well as promote efficient cellular uptake by endocytosis and/or via membrane pore formation [60–62], have been extensively studied as drug delivery vectors for nucleic acid based therapies including plasmid DNA and siRNA/antisense oligonucleotides [22,23,31,47,49,53,63]. The formed complexes of PAMAM dendrimers with nucleic acids are usually termed “polyplexes” or “dendriplexes” that can more efficiently deliver nucleic acids into cells.

Non-viral drug delivery systems, including PAMAMs, aside from their drug delivery-enhancing capacity, should ideally be biologically inert [22,23,31]. In the last decade or so, an emerging theme arising from several micro-array based global gene expression profiling studies [30,64–69] and protein signaling studies [37,70–72], have highlighted that even so called ‘biocompatible” polymers can exert intrinsic biological activity such as modulation of global gene expression and interference with key cell signaling cascades (for reviews see [22,23,31,73]. A study of the biological effects of drug delivery systems will not only have a bearing on their nanotoxicology and clinical safety but importantly, may also highlight potentially novel biological actions that could be exploited therapeutically [37]. Thus, a common consensus in the nanotechnology field is that drug delivery system-induced biological changes to living cells need to be profiled in detail both *in vitro* and *in vivo* and their clinical significance determined. Recent literature highlighting these biological properties of PAMAMs is reviewed herein.

3. Biodistribution and pharmacokinetics of PAMAMs *in vivo*

To assess the likely biological actions exerted by naked PAMAMs and their possible impact on a given organ/tissue/cell system, it is necessary to study the likely fate of PAMAMs in the body following administration via different routes.

Despite the reported enhanced pharmacokinetic profiles of many drugs and marketed formulations when conjugated to PAMAM dendrimers [47,74,75], the pharmacokinetic modeling of naked PAMAMs (without a drug cargo) administered via different routes has not been extensively reported. It should be noted that the fate of drugs carried by PAMAM dendrimer delivery system does not necessarily reflect that of PAMAM dendrimer itself as the latter can dissociate and have a fate that is different from the drug. Due to the potential diversity of PAMAM dendrimer structure, size, and surface charge that can be generated by modifications to their core and/or surface, their ability to cross cellular membranes will vary accordingly, and consequently their pharmacokinetic profiles are expected to be widely versatile.

Generally, it is known that *in vivo* administration of nanoparticulate drug delivery systems, including PAMAMs, usually results in their biodistribution into tissues of the reticuloendothelial system such as the spleen, lung, liver, heart and kidney [76]. There is also evidence that PAMAMs can traverse the blood–brain barrier and in the CNS, neutral G4 PAMAMs can localise to microglia and astrocytic inflammatory cells of the brain [77]. In a study of ^{14}C-labelled PAMAMs, Roberts et al (1996) also showed extremely high accumulation of cationic G5 and G7 in the pancreas- a finding that might have clinical implications in diabetes. It was generally found that cationic dendrimers were rapidly removed from blood circulation (<2h) and rapidly eliminated via the kidneys into the urine [78].

In key studies lead by the Ghandehari group [60,79], the biodistribution and pharmacokinetics of three generations (G5, G6, and G7) of the neutral PAMAMs were characterized in tumour-bearing mice and modelled into a two-compartmental distribution model with a central link to two tumour serial compartments. There were significant differences in the pharmacokinetic primary parameters, including elimination and renal clearances among the three PAMAM generations that was dependent on polymer size/-generation. As the hydrodynamic size (or generation) of the PAMAMs increased, the elimination clearance decreased linearly in a log scale. This was also in agreement with the renal clearance when urine data were analyzed, with lower reported values than the overall elimination clearance suggesting a side pathway of elimination such as through the liver and spleen. Tomalia et al. [54] demonstrated that MRI images of mice injected with different generations of cationic PAMAMs showed that as the PAMAM generation increases, its predominant elimination pathway shifts from the kidneys (PAMAMs < G5) to the liver (PAMAMs > G5). Thus, lower generations of cationic PAMAMs (G2 to G5) demonstrated a predominant accumulation in the kidney compared to the higher generations (G6 to G9) which tended to be trapped in the reticuloendothelial system of the liver and spleen [80].

As a main elimination process within the kidneys, glomerular filtration was found to play a major role in the renal elimination of PAMAMs. With a cut-off diameter of ~5.0 nm for the majority of pores [81], the glomerulus selectively allows the filtration of polymers below this threshold. This size-dependent glomerular filtration was evident with PAMAMs of hydrodynamic radius of around 2.0 nm being filtered easily through the kidney compared to PAMAMs of larger hydrodynamic radius of around 4.0 nm being close to the glomerular filtration threshold leading to lower renal clearances and longer blood exposure. Therefore, this polymer size-dependent pharmacokinetics was also reflected on polymer blood exposure (dose normalized AUC) which was increased when PAMAM generation increased from G5 to G7, suggesting a longer half-life predicted for larger polymers. For example, G7-OH was shown to have the longest plasma circulation retention compared to lower generations, resulting in its overall distribution almost equally to all body organs [79,82]. It is noteworthy to mention, that the surface chemistry of PAMAMs also plays a significant role in determining their biodistribution and pharmacokinetics. For example, radiolabeled charged PAMAMs injected intravenously or intraperitoneal in rats have been shown to readily clear from the circulation and accumulate significantly in the liver, with anionic taking a longer time to clear than cationic PAMAMs [83]. Surface modification of these charged PAMAMs to render them hydrophilic, e.g., by PEGylation, could limit their hepatic clearance and consequently prolong their half-life [84,85]. A careful selection of the degree of PEGylation not only improves the pharmacokinetics of PAMAMs, but also reduces their toxicity especially for the cationic constructs [86,87].

It is thought that PAMAM dendrimer architecture also has an influence on its passage through cellular membranes, and consequently their pharmacokinetic profiles. The extent of branching, ability to deform, and the possibility of being globular have been shown to be correlated with PAMAMs biodistribution [8,55,83]. In contrast to linear polymers, which can reptate through porous membranes (i.e. through a motion analogous to snakes slithering

one through another), PAMAM dendrimers have been shown to lose their ability to deform and get through membranes as their size/generation increases [88–91]. Thus, PAMAMs at higher generations show structure rigidity that limits their ability to deform and pass through fenestrated membranes such as those existing in the liver and kidney tissues, influencing their elimination/clearance. A possible solution to reducing rigidity is to decrease dendrimer generation; alternatively, a direct reduction of dendrimer internal branching such as in the production of "activated" PAMAMs through post-synthesis solvolytic degradation of higher generations is known to lead to nanostructures that are more efficient as transfection agents [57–58]. However, the role of particle rigidity/elasticity requires further study as in some cases rigid nanostructures may offer better cell uptake properties and additionally, rigidity/elasticity may be of less importance than particle size and shape in determining membrane permeability (for reviews see [92,93]).

In non-eliminating tissues and organs, the pharmacokinetics of PAMAM dendrimers have also been correlated with their physiochemical properties especially their size/generation and surface charge. Extravasation and accumulation of PAMAMs into tumour tissues have been shown to be greater compared to extravasation to normal tissues [79,94]. Indeed, this selective biodistribution or enhanced permeability and retention (EPR) effect in particular has been the basis of using nanoparticles, including PAMAM dendrimers, as potential delivery carriers for many anti-cancer agents [95–97].

Beyond parenteral administration, the absorption process following extravascular administration has been separately characterized. To model its absorption through the intestinal epithelial barrier, the permeability of PAMAM dendrimers was characterized *in vitro* across Caco-2 cell monolayer and found to be transported through a paracellular and energy-dependent process, e.g., adsorptive endocytosis [8,98–101]. PEGylation of anionic PAMAMs, however, has been shown to reduce its paracellular transport [102]. Although cationic and anionic PAMAMs have been shown *in vitro* to increase the paracellular transport of some drugs by opening tight junctions, most likely by disrupting the tight junctional proteins occludin and actin [60], this phenomenon was not observed *in vivo* where PAMAMs were reported to enhance camptothecin uptake independently of tight junction opening [103]. The role of membrane transporters such as p-glycoprotein on the transport of PAMAM dendrimers is yet to be confirmed, as the data are inconclusive [8,60,104–106].

At the cellular level, membrane association, cell uptake and intracellular fate of PAMAMs appears also to be dependent on dendrimer concentration and on the physico-chemical properties of surface charge and molecular weight as well as the cell-type being investigated. Although a comprehensive review of the cellular uptake mechanisms is beyond the scope of this review (for a recent review see [59], generally, PAMAMs enter cells by some form of vesicular transport (e.g. endocytosis or macropinocytosis) and/or via membrane pore formation [59,60,62,107,108]. The pore-formation theory arises from studies showing that higher generation dendrimers (>G4) can cause pore formation and strip lipids from the membranes, whereas low generation dendrimers (<G5) have been found to intercalate or adsorb to membrane surfaces (for a review see [59]. Cationic PAMAMs may also be substrates for efflux transporters such as p-glycoprotein as demonstrated in drug resistant cancer cells [107], though this requires further study. The precise PAMAM cell uptake and trafficking mechanisms are strongly dependent on cell type as significant differences in cell uptake mechanisms and exocytosis of a G4 PAMAM were noted in multidrug resistant breast cancer (MCF-7) cells compared to their sensitive wild type cells [107]. For example, greater macropinocytosis, exocytosis and lysosomal degradation of G4 PAMAM was reported in drug resistant cells than wild-type MCF-7 cells [107]. The intracellular fate of PAMAMs or their breakdown products is less well studied. PAMAMs are thought to escape endosomal/lysosomal compartments (possible via a proton sponge effect) and have been reported to locate to a variety of intracellular sites including the cytosol, nucleus, mitochondria, Golgi complex and the endoplasmic reticulum [59,107,109–110]. Thus, their location within the many sub-cellular organelles might aid understanding of the diverse biological actions possible with PAMAM dendrimers. Indeed, since PAMAMs are thought to be biodegradable; knowledge of both the intracellular fate of the intact dendrimer nanostructures as well as their degradation products requires greater study as this information will undoubtedly be important in correlating their intracellular location with biological activity (e.g. it could help distinguish between the relative contribution of PAMAM-mediated changes in mitochondrial versus nuclear gene expression).

The above studies suggest that the cellular uptake and trafficking, as well as the *in vivo* biodistribution and pharmacokinetics of PAMAMs, are significantly influenced by the dendrimer generation (size) and surface chemistry. Furthermore, it should be noted that conjugation or attachment of a drug can alter the pharmacokinetics of PAMAMs; for example nucleic acids complexed with dendrimers (forming so-called "dendriplexes") are more stable and generally exhibit longer circulation times [76]. Indeed, the *in vivo* kinetics of disassociation of PAMAMs from their conjugated drug moieties, the subsequent fate of intact and breakdown products of PAMAMs as well as their biological consequences remain poorly understood and require further study.

4. Innate properties beyond drug delivery: Biological and toxicological actions of PAMAMs

An emerging consensus is that beyond their drug delivery effects, PAMAMs also exert several biological/toxicological actions ranging from molecular effects (such as their impact on gene expression and on cell signalling proteins) to bulk macroscopic effects such as haemolysis and cell death (see Fig. 2 for a summary). These are discussed further in the proceeding sections.

4.1. PAMAM-induced gene expression and the concept of "toxicogenomics"

Toxicogenomics is a branch of (nano) toxicology that examines the effects of a nanomaterial on (global) gene expression through a study of cellular transcriptomics e.g. via using micro-array based gene expression assays [23,31]. In this way, our laboratory was the first to report on the ability of several drug delivery systems such as lipid transfection agents (e.g. Lipofectin and Oligofectamine) [64,66], linear and branched polyethyleneimine [22], polypropyleneimine dendrimers [65], and PAMAMs [30] to elicit multiple gene expression changes in human cells- findings that have been confirmed subsequently by others (see Table 1 for a summary of studies showing gene expression changes elicited by PAMAMs).

Drug delivery systems including commercial cationic liposomal formulations, generally elicited multiple gene expression changes in the absence of causing detectable direct DNA damage in cells [31,66]. In our toxicogenomics evaluation of PPI and PAMAM dendrimers, we found that the nature and extent of gene expression changes induced by these drug delivery systems were dependent on the dendrimer generation, dendrimer branching architecture, surface charge, the type of the nucleic acid present in dendriplexes and on the type of cell being studied [22,30,31,65]. Drug delivery system-induced gene changes were observed in *in vitro* cell culture

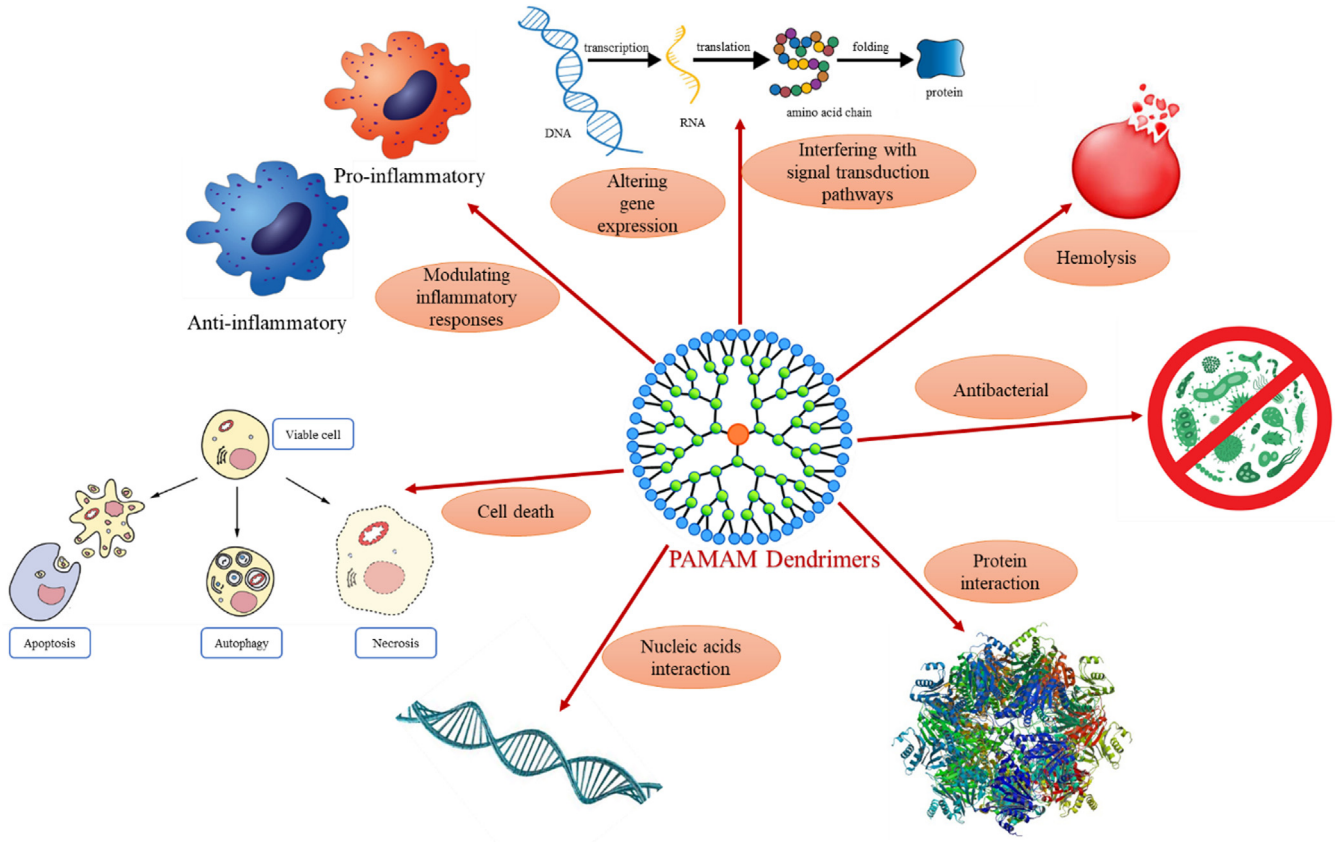

Fig. 2. A summary of biological or toxicological effects of PAMAM dendrimers: A schematic showing the potential ability of PAMAMs in altering gene expression, interfering with signal transduction pathways, inducing hemolysis, producing antibacterial effects, interacting with cellular proteins and nucleic acids, inducing cell death, and modulating inflammatory responses.

systems as well as in an animal model (e.g. mouse xenograft for linear and branched polyethyleneimine) [22]. Furthermore, drug delivery system-induced gene expression changes appear to occur after both acute and chronic exposure of delivery systems though the nature of the genes affected are likely to be different.

Interestingly, in the first report of PAMAM-mediated gene expression, two commercially available G6 PAMAM dendrimer delivery systems, Polyfect (PF) and Superfect (SF), were found to elicit marked gene expression changes in human epidermoid carcinoma (A431) cells (see also Table 1, Fig. 3) [30]. Using a high-density microarray chip housing 20,000 gene spots, a 2-fold gene expression change (up- and down-regulation) in 1749 genes were observed for SF and only 1103 genes for PF after 4 h incubation with cells. These two cationic amino-terminated G6 PAMAMs, differing only in their structural branching architecture, were reported to have opposing effects on the expression of epidermal growth factor receptor (EGFR) implying that PAMAM structural architecture had an important influence on the ability of PAMAMs to elicit gene expression changes in cells. Although the effect of PAMAM surface chemistry was not directly investigated in this study, the authors noted that upon complexation of PF with DNA, a concomitant reduction in the zeta potential (a measure of surface charge) was accompanied with a significant reduction in the number of gene changes (greater than 30%) induced by the PF-DNA dendriplexes. However, unexpectedly PF-siRNA dendriplexes exhibited a 70% increase in the number of gene expression changes [30] implying that the nature of the nucleic acid was an important determinant of this biological effect and further implied that each PAMAM-drug complex might exhibit a unique "gene signature" as it is likely seen by cells as a separate entity from the PAMAM carrier alone.

The gene expression effects observed by Hollins et al (2007) were supported by a later study that concluded G5 cationic PAMAM dendrimers could also alter the expression patterns of multiple genes in HeLa cells in a structure-dependent manner, with a more pronounced impact seen with activated PAMAMs compared to non-activated ones [68]. As to the nature of the genes/gene ontologies affected by PAMAM dendrimers, these are mostly implicated in cell survival, proliferation, nucleic acid binding, transcription activity, and some other biological processes (see also Table 1 that gives a comprehensive listing of the gene changes reported with PAMAMs).

The surface chemistry of PAMAM dendrimers can strongly influence their toxicogenomic effects. The effect of surface charge was reported in primary human bronchial epithelial cells, PBECs, where cationic, but not anionic, PAMAMs downregulated genes related to cell cycle and cell division mediated by NF-κB [67]. At low-dose exposure, an upregulation was reported in genes implicated in immune responses, such as TNF-R and IL-1β, as well as genes involved in cell migration and disassembly of the extracellular matrix, such as MMP3 and MMP9 [67]. The effect of PAMAMs on inflammatory responses is further discussed in a subsequent section (see below).

The nature and extent of gene expression changes elicited by PAMAMs (see Table 1 for list), even at low doses that do not show overt cell toxicity, may allow early prediction of subsequent gross biological and toxicological effects especially in terms of apoptosis. Furthermore, gene changes may be considered beneficial or detrimental in the context of the intended purpose for which PAMAMs might be used. In the context of using PAMAMs for delivering siRNA, Hollins et al (2007) showed that PAMAM-induced pleiotropic gene expression could be detrimental as they may add to the

Table 1
Gene expression changes induced by naked PAMAM dendrimers. Genes reported to be upregulated or downregulated are shown.

Gene	Protein encoded	PAMAM used	Generation	Model	Methodology	Effect of PAMAMs [1]		Reference(s)
ABCB1	ATP-binding cassette, subfamily B (MDR/TAP), member 1	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑		Kuo et al [68]
ANLN	Anillin actin binding protein	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
ARHGAP11A	Rho GTPase activating protein 11A	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
ARNTL2	Aryl hydrocarbon receptor nuclear translocator like 2	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR		↓	Kuo et al [68]]
ART4	ADP-ribosyltransferase 4 (Dombrock blood group)	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR		↓	Kuo et al [68]
ASPM	Assembly factor for spindle microtubules	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
ATF3	Activating transcription factor 3	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
AURKB	Aurora kinase B	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
BIRC5	Baculoviral IAP repeat-containing protein 5	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
BRCA1	Breast cancer type 1 susceptibility protein	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
BTG2	B-cell translocation gene 2	Cationic	G3, G4	THP-1 (acute monocytic leukemia) U937 (histiocytic lymphoma)	RT-PCR	↑		Janaszewska et al [171]
BUB1	Mitotic checkpoint serine/threonine kinase	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
BUB1B	Mitotic checkpoint serine/threonine kinase B	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
C1orf112	Chromosome 1 open reading frame 112	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
C5orf23	Chromosome 5 open reading frame 23	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑		Kuo et al [68]
CASC5	kinetochore scaffold 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CCDC80	Coiled-coil domain containing 80	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
CCNB1	Cyclin B1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CDC20	Cell division cycle protein 20	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CDC25C	Cell division cycle 25C	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CDC45	Cell division cycle 45	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CDCA2	Cell division cycle associated 2	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CDCA5	Cell division cycle associated 5	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CDCA8	Cell division cycle associated 8	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CDK1	Cyclin-dependent kinase 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
CDKN3	Cyclin-dependent kinase inhibitor 3	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CENPF	Centromere protein F	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CEP55	Centrosomal protein 55	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CHD4	Chromodomain helicase DNA binding protein 4	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR		↓	Kuo et al [68]
CHERP	Calcium homeostasis endoplasmic reticulum protein	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑		Kuo et al [68]
COL5A1	Collagen type V alpha 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
COL6A2	Collagen type VI alpha 2	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
CRADD	Death domain-containing protein	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CSF1	Colony stimulating factor 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
CSF2	Colony stimulating factor 2	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
CX3CL1	C-X3-C motif chemokine ligand 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
CXYorf3	Homo sapiens chromosome X and Y open reading frame 3	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR		↓	Kuo et al [68]
DLGAP5	DLG associated protein 5	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
DPP4	Dipeptidyl peptidase-4	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
E2F1	E2F transcription factor 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
EGFR	Epidermal growth factor receptor	SF and PF cationic	G6	Human epidermoid carcinoma A431 cell line	Microarray, RT-PCR, Western Blot	↑	↓	Hollins et al [30]
		SF cationic	G6	human embryonic kidney cells	Western Blot	↑		Akhtar et al [70]
		PF cationic	G6	Human embryonic kidney cells	Western Blot		↓	Akhtar et al [71]
		SF and PF cationic	G6	Male Wistar rats	Western Blot	↑	↓	Akhtar, Al-Zaid, et al [72]
		Cationic	G4, G5, G6, G7	Primary rat aortic smooth muscle cells	Western Blot		↓	Akhtar, El-Hashim, et al [120]
		Cationic	G6	Primary rat aortic smooth muscle cells	Western Blot		↓	Akhtar et al [37]
ENST00000370419	Regulating synaptic membrane exocytosis protein 1 (RIM 1)	Non-activated, cationic	G5	HeLa cancer cells	Microarray	↑		Kuo et al [68]
ErbB2	Erythroblastic oncogene B2	Cationic	G4, G5, G6, G7	Primary rat aortic smooth muscle cells	Western Blot		↓	Akhtar, El-Hashim, et al [120]
EREG	Epiregulin (EPR)	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
ERK1/2	Extracellular signal-regulated protein kinase	SF cationic	G6	Human embryonic kidney cells	Western Blot	↑		Akhtar et al [70]
		PF cationic	G6	Human embryonic kidney cells	Western Blot		↓	Akhtar et al [71]
		SF and PF cationic	G6	Male Wistar rats	Western Blot	↑	↓	Akhtar, Al-Zaid, et al [72]
		Cationic	G4, G5, G6, G7	Primary rat aortic smooth muscle cells	Western Blot		↓	Akhtar, El-Hashim, et al [120]
		Cationic	G6	Primary rat aortic smooth muscle cells	Western Blot		↓	Akhtar et al [37]
ESPL1	Extra spindle pole bodies like 1, separase	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
ETS1	ETS proto-oncogene 1, transcription factor	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
ETS2	ETS proto-oncogene 2, transcription factor	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
FAM129A	Protein niban	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
FANCA	Fanconi anemia complementation group A protein	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
FANCD2	Fanconi anemia complementation group D2 protein	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
FAT2	FAT atypical cadherin 2	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
FOLH1	Folate hydrolase (prostate-specific membrane antigen) 1	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑		Kuo, Liou and Chiu [68]
FOXM1	Forkhead box M1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
FOXO1	Forkhead box O1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
GIPR	Gastric inhibitory polypeptide receptor	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑		Kuo, Liou and Chiu [68]
HJURP	Holliday junction recognition protein	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]

(*continued on next page*)

Table 1 (*continued*)

Gene	Protein encoded	PAMAM used	Generation	Model	Methodology	Effect of PAMAMs [1]	Reference(s)
HMMR	Hyaluronan mediated motility receptor	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
HMOX1	Heme oxygenase 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
HSF1	Heat shock transcription factor 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
ICAM1	Intercellular adhesion molecule 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
IGF2	Insulin like growth factor 2	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
IL-1β	Interleukin 1 beta	Cationic	G4	Primary bronchial epithelial cells	mRNA Sequencing	↑	Feliu et al [67]
		Anionic and neutral	G5	Mice pancreatic tissues	RT-PCR	↓	Tang et al [170]
				LPS-stimulated mouse peritoneal macrophages		↓	
		Cationic	G3, G4	THP-1 (acute monocytic leukemia) U937 (histiocytic lymphoma)	RT-PCR	↓	Janaszewska et al [171]
IL-10	Interleukin 10	Anionic and neutral	G5	Mice pancreatic tissues	RT-PCR	–	Tang et al [170]
				LPS-stimulated mouse peritoneal macrophages		–	
IL-32	Interleukin 32	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
IL-6	Interleukin 6	Anionic and neutral	G5	Mice pancreatic tissues	RT-PCR	↓	Tang et al [170]
				LPS-stimulated mouse peritoneal macrophages		↓	
KCNJ10	Potassium inwardly rectifying channel, subfamily J, member 10	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↓	Kuo et al [68]
KDM5B	Lysine demethylase 5B	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
KIF11	Kinesin-like protein KIF11	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
KIF18A	Kinesin-like protein KIF18A	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
KIF20A	Kinesin-like protein KIF20A	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
KIF2C	Kinesin-like protein KIF2C	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
KIFC1	Kinesin-like protein KIFC1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
KRT15	Keratin 15	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
LAMA4	Laminin subunit alpha-4	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
LMNB1	Lamin-B1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
LTB	Lymphotoxin beta	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
MAD2L	Mitotic spindle assembly checkpoint protein	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
MATN1	Cartilage matrix protein precursor (Matrilin-1)	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↓	Kuo et al [68]
MCM10	Minichromosome maintenance complex component 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
MCM3	Minichromosome maintenance complex component 3	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
MCM4	Minichromosome maintenance complex component 4	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
MKI67	Marker of proliferation Ki-67	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
MMP3	Matrix metalloproteinase-3	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
MMP9	Matrix metalloproteinase-9	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
MTHFD1	Methylenetetrahydrofolate dehydrogenase 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
MTFR2	Mitochondrial fission regulator 2	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
MYBL2	Myb-related protein B	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
MYC	MYC proto-oncogene	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
NCAPD3	Non-SMC condensin complex subunit D3	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
NCAPG	Non-SMC condensin complex subunit 3	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
NCAPH	Non-SMC condensin complex subunit H	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
NFKB1	Nuclear factor Kappa B subunit 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
NFKBIA	Nuclear factor Kappa B inhibitor alpha	Cationic	G3, G4	THP-1 (acute monocytic leukemia) U937 (histiocytic lymphoma)	RT-PCR	↓	Janaszewska et al [171]
		Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
NOS1	Nitric oxide synthase 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
NRP1	Neuropilin-1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
NUPR1	Nuclear protein 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
NUSAP1	Nucleolar and spindle-associated protein 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
OR2W3	Olfactory receptor family 2 subfamily W member 3	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑	Kuo et al [68]
P38 MAPK	P38 mitogen-activated protein kinases	PF cationic	G6	Human embryonic kidney cells	Western Blot	↓	Akhtar et al [71]
		SF and PF cationic	G6	Male Wistar rats	Western Blot	↑ ↓	Akhtar, Al-Zaid, et al [72]
PDE11A	Phosphodiesterase 11A transcript variant 4	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↓	Kuo et al [68]
PDGFB	Platelet derived growth factor subunit B	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
PHF5A	PHD-finger domain protein 5A	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑	Kuo et al [68]]
PLK1	Polo-like kinase 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
POLQ	DNA polymerase theta	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
PRC1	Protein regulator of cytokinesis 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
PRKDC	DNA-dependent protein kinase	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
PTGFR	Prostaglandin F receptor (FP), transcript variant 2	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↓	Kuo et al [68]
PTPRR	Protein tyrosine phosphatase, receptor type, R	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑	Kuo et al [68]
PTPRZ1	Receptor-type tyrosine-protein phosphatase zeta	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
RACGAP1	Rac GTPase activating protein 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
RAD51AP1	RAD51 associated protein 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]
RB1	Retinoblastoma gene	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
RBP1	Retinol binding protein 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
RELA	Transcription factor p65	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑	Feliu et al [67]
RGS9BP	Regulator of G protein signaling 9 binding protein	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↓	Kuo et al [68]
ROCK	Rho-associated protein kinase	Cationic	G6	Primary rat aortic smooth muscle cells	Western Blot	↓	Akhtar et al [37]
RRM2	Ribonucleoside-diphosphate reductase subunit M2	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↓	Feliu et al [67]

Table 1 (*continued*)

Gene	Protein encoded	PAMAM used	Generation	Model	Methodology	Effect of PAMAMs [1]		Reference(s)
RSU1	Ras suppressor protein 1, transcript variant	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑		Kuo, Liou and Chiu [68]
SFRP1	Secreted frizzled-related protein 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
SHCBP1	SHC binding and spindle associated 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
SLC8A1	Sodium/calcium exchanger 1	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR		↓	Kuo et al [68]
SORL1	Sortilin related receptor 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
SPAG5	Sperm-associated antigen 5	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
Src	Proto-oncogene tyrosine-protein kinase	Cationic	G4, G5, G6, G7	Primary rat aortic smooth muscle cells	Western Blot		↓	Akhtar, El-Hashim, et al [120]
TCF3	Transcription factor 3	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
TGFβ-1	Transforming growth factor beta 1	Anionic and neutral	G5	Mice pancreatic tissues	RT-PCR	–		Tang et al [170]
				LPS-stimulated mouse peritoneal macrophages		–		
TGFβR-1	Transforming growth factor beta receptor 1	Anionic and neutral	G5	Mice pancreatic tissues	RT-PCR		↓	Tang et al [170]
				LPS-stimulated mouse peritoneal macrophages			↓	
TICRR	TOPBP1 interacting checkpoint and replication regulator	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
TIMP2	Tissue inhibitor of metalloproteinases 2	Cationic	G4	Primary bronchial epithelial cells	mRNA Sequencing	↑		Feliu et al [67]
TNF-α	Tumor necrosis factor alpha	Anionic and neutral	G5	Mice pancreatic tissues	RT-PCR		↓	Tang et al [170]
				LPS-stimulated mouse peritoneal macrophages			↓	
		Cationic	G4	Primary bronchial epithelial cells	mRNA Sequencing	↑		Feliu et al [67]
		Cationic	G3, G4	THP-1 (acute monocytic leukemia) U937 (histiocytic lymphoma)	RT-PCR	↑		Janaszewska et al [171]
TNF-R	Tumor necrosis factor receptor	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
TOP2A	DNA topoisomerase II alpha	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
TP53	Tumor protein 53	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing	↑		Feliu et al [67]
TPX2	Microtubule nucleation factor	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
TRIM49	Tripartite motif-containing 49	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR	↑		Kuo et al [68]
TTK	Dual specificity protein kinase	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67]
UGT8	UDP glycosyltransferase 8	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR		↓	Kuo et al [68]
USP29	Ubiquitin specific peptidase 29	Non-activated, cationic	G5	HeLa cancer cells	Microarray, RT-PCR		↓	Kuo et al [68]
VRK1	VRK serine/threonine kinase 1	Cationic	G4	Primary bronchial epithelial cells	mRNA sequencing		↓	Feliu et al [67][1]

[1] ↑ refers to a significant increase in gene expression, ↓ refers to a significant decrease in gene expression as reported by the authors.

"off-target" effects already observed with these gene silencing agents [30]. In contrast, these authors also showed that by carefully selecting a delivery system that additionally inhibited the actual gene targeted by siRNA (as exemplified by EGFR), gene silencing activity could be enhanced and thus, in this case PAMAM-induced modulation of gene expression could be considered beneficial. In any case, for the appropriate selection of a suitable delivery system, including specific PAMAMs, we recommend toxicogenomics screening as part of their assessment for a given intended clinical or non-clinical purpose. However, a question that remains is "at what time point of exposure should toxicogenomics studies be performed?". In most experimental settings, the time selected is that which is matched to the time chosen for determining a given biological end-point e.g same time point as when you examine target gene silencing in cells or animals. However, this does not always provide answers on the long-term effects of these drug delivery systems and their likely clinical safety. Indeed, an important research question that remains poorly understood, is the precise temporal profile of the gene changes elicited by PAMAMs. It would also be important to delineate the mechanisms involved in eliciting acute versus chronic gene changes and whether the latter involves persistence of the same genes from acute effects or mediated by different genes resulting from multiple cascade events subsequent to the initial (acute) gene expression changes. As to the mechanism, we and others have noted specific gene and signalling changes in the acute *in vitro* cell setting are mediated via PAMAM-induced oxidative stress mechanisms [70,71,110,112], though this needs further verification in other models *in vitro* and *in vivo*.

4.2. Modulation of signal transduction pathways

With the caveat that not every single gene expression change will lead to a corresponding change in the encoded protein, actual measured cellular mRNA and protein levels mostly show reasonable correlation [113]. Thus, a natural corollary to drug delivery system-induced gene changes is that these will ultimately lead to cellular changes in protein levels including those involved in cell signalling and indeed, this is the case with PAMAMs.

PAMAM dendrimers can strongly interact with and/or disrupt key cell signaling cascades *in vitro* and *in vivo*, thereby modulating cellular communication processes that control the overall cell function and organization (Fig. 3, Fig. 4). For example, EGFR signalling is critical for cell growth, proliferation, differentiation, migration and survival of cells (reviewed in [114,115]). Cationic PAMAM dendrimers were found to alter the EGFR-ERK1/2 cellular signal transduction pathway in a dose and time-dependent manner in human embryonic kidney cells, that was also confirmed *in vivo* in animal models [70–72]. The use of antioxidants reversed the impact of PAMAMs on the investigated cell signaling pathway, which reveals that PAMAMs modulate cell signaling pathways via an oxidative-stress-dependent mechanism [70–72].

Similar to their differential effects on EGFR gene expression *in vitro* [30], SF and PF dendrimers also had opposing actions on EGFR signaling transduction pathway *in vivo* [70,71] - not only implying that dendrimer architecture influences gene and protein signaling changes alike but further suggested that PAMAMs might be considered as a new class of EGFR receptor modulators allowing both stimulation (ligand-like) or inhibition (receptor antagonist-like actions) [71]. ERK1/2 and p38 MAP kinase signaling cascades were also modulated by PAMAM dendrimers; since these are important cell signaling molecules that converge and relay signals from multiple cell surface receptors, their dysregulation by PAMAMs might have important implications regarding the use of these polymers in nanomedicine [72].

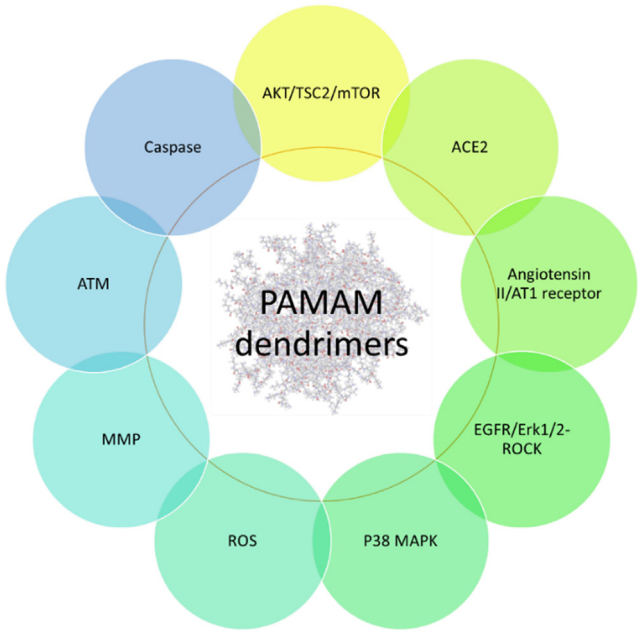

Fig. 3. Examples of cell signaling molecules/pathways modulated by PAMAM dendrimers. These include ACE2 [122], EGFR/ERK1/2-ROCK [71,115], p38 MAP kinase [72], ROS [71,111–112,153,145–147], renin-angiotensin pathway [122], and AKT-TSC2-mTOR [124], MMP [67,148–149], ATM [148–149], Caspase [71,112,153], and AKT-TSC2-mTOR [124].

Signaling via Angiotensin II, a key peptide of the renin-angiotensin system (RAS) is vital for blood pressure regulation and, partly through its ability to transactivate EGFR signaling [116–120], has been implicated in several pathologies including cardiovascular diseases [114,119]. In a systematic study of a series of PAMAM generations (G4, G5, G6, and G7) and different surface chemistries (cationic, anionic, and neutral), cationic PAMAMs had the highest inhibitory effect on Angiotensin II/AT_1 receptor-mediated transactivation of EGFR and ErbB2, a second member of the EGFR family of receptors, in a dose and time-dependent fashion in primary vascular smooth muscle cells. There was a strong correlation of these biological effects with dendrimer generation as the inhibition of Ang II-mediated transactivation of EGFR/ErbB2 increased upon treatment with higher generations (G7 > G6 > G5 > G4) of PAMAMs. This systematic evaluation of dendrimer generation and surface chemistry on EGFR cell signaling further provided proof of the important role that physicochemical properties played in the biological actions of drug delivery systems [120]. Moreover, mechanistic studies with cationic G5 PAMAM confirmed suppression of Ang II-mediated transactivation of EGFR/ErbB2 occurred through inhibition of the non-receptor tyrosine kinase (Src) [120] implying that Src signaling may also be the target for PAMAMs. Further, it is known that upregulated Src-dependent EGFR signaling via downstream effectors like ERK1/2 and Rho kinases (ROCK) can mediate diabetes-induced vascular complications [37,114,121]. In an animal model of diabetes, it was shown that PAMAM dendrimers can inhibit the EGFR-ERK1/2-ROCK signaling pathway *in vivo* in a generation and a sur-

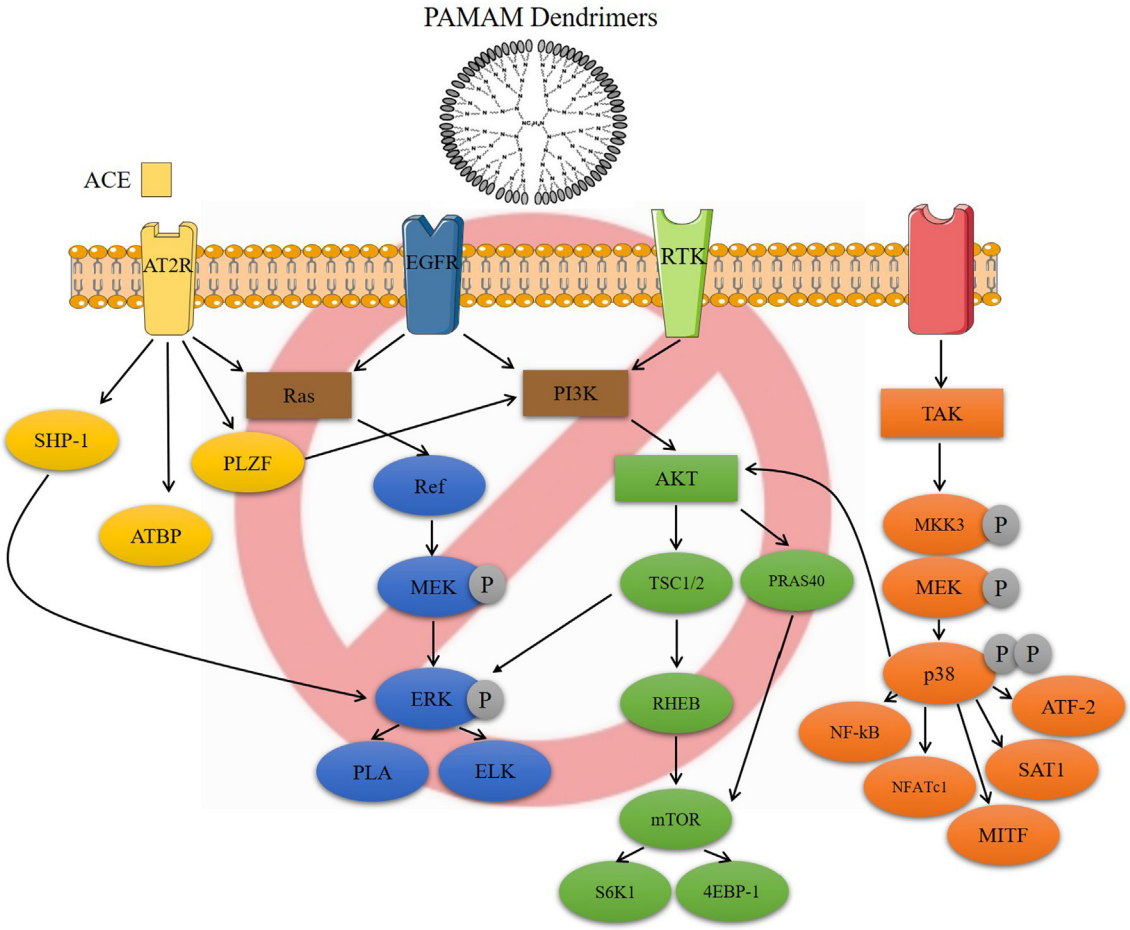

Fig. 4. Examples of the main signalling pathways modulated by PAMAM dendrimers: these include EGFR/ERK1/2 [71,115], p38 MAP kinase [72], renin-angiotensin pathway [122], and AKT-TSC2-mTOR [124].

face chemistry dependent manner (cationic > anionic > neutral) [37]. This was accompanied by a mitigation of the vascular dysfunction associated with diabetes, and thereby raises hope in utilizing PAMAMs as possible treatments for diabetes-induced vascular complications [37] (see also perspectives section).

Cationic PAMAM can also bind and inhibit angiotensin converting enzyme type 2 (ACE2) [122], thereby dysregulating the renin-angiotensin pathway through blockade of its counter-regulatory arm comprising ACE2-Angiotensin-(1-7)- Mas receptor that generally opposes the actions of Ang II [114,123]. In the absence of ACE2- the same molecule that is known to facilitate cellular entry of SARS-COV-2 virus that induces pulmonary distress syndrome (a form of lung injury) in COVID-19 patients (for a recent review see [123], Ang II actions will predominate and in pulmonary pathology these are largely detrimental as they mediate lung injury [123]. Inhalation of a cationic, but not anionic, G5 PAMAM dendrimer in mice led to induction of lung injury through inhibition of ACE2 and raised Ang II levels [122]. Elevated Ang II levels occurred presumably as a compensatory mechanism, but surprisingly this was in the absence of changes in ACE levels [122]. It was hypothesised that blocking Ang II signaling with Losartan, an AT_1 receptor antagonist, could rescue dendrimer-induced lung injury. Taken together, the above studies imply that PAMAM dendrimers are important modulators of RAS as they can likely inhibit both "arms" of the RAS signaling cascade depending on the tissue or organ system being exposed- this fact will be dependent on the route of administration and the resulting biodistribution and pharmacokinetics PAMAMs.

In the context of lung toxicity, it was also found that cationic PAMAMs induce autophagic cell death mediated by the dysregulation of the Akt-TSC2-mTOR signaling pathway that also mediates acute lung injury [124]. A set of other pathways related to cell apoptosis and autophagy are also deregulated by PAMAM dendrimers and will be discussed in the subsequent sections below.

4.3. Interactions with other sub-cellular components or molecules

PAMAM dendrimers tend to interact and bind with cellular and subcellular structures and proteins depending on the surface chemistry and generation of the used PAMAMs. These interactions may either change the function of the substrate in a desirable manner or induce cellular toxicity [37,125]. Several potentially beneficial actions of PAMAMs have been reported in this context.

Amylin or human islet amyloid polypeptide (hIAPP), that is co-secreted with insulin from pancreatic beta-cells, is important for glucose homeostasis. However, amyloid deposits of this peptide in the islets of Langerhans can lead to beta-cell toxicity and type-2 diabetes. Gurzov et al showed that neutral G3 PAMAMs can effectively prevent the aggregation of hIAPP and mitigate hIAPP-mediated toxicity in pancreatic MIN6 and NIT-1 cell lines as well as in mouse islets [126]. Mechanistically, PAMAM dendrimers prevented the aggregation of hIAPP by stopping its polymerization through reducing interpeptide contacts and hydrogen bonds [126].

The surface charge/chemistry of PAMAM dendrimers is a major determinant of their interaction with cellular components. For example, cationic and anionic PAMAMs showed opposing effects on the assembly and function of tight junctions in human colorectal adenocarcinoma cell line (Caco-2) [127]. Interestingly, anionic PAMAMs failed to open tight junctions, whereas cationic PAMAMs altered tight junctions allowing small molecule transportation. The reported mechanism behind this effect was linked partially with the PLC-dependent signaling pathway in addition to intracellular calcium release caused only by cationic PAMAMs [127]. On the other hand, cationic PAMAM dendrimers blocked the pore-forming B components of the binary anthrax toxin (PA63) and clostridium botulinum C2 toxin (C2IIa) at low concentrations [128]. Therefore, by blocking these pores, cationic PAMAMs inhibited transporting of the A components from endosomes to the cytosol of target cells. These data show a novel biological effect of PAMAM dendrimers in which they can protect human target cells from the toxicity of binary toxins released from pathogenic bacteria [128]. PAMAM dendrimers also showed anticoagulant activities through binding with polyphosphates and modulating activation of the intrinsic pathway of coagulation *in vitro*. Low generation PAMAMs inhibited clotting in a polyphosphate (polyP) 60- and 130-dependent mechanism, while higher generations reversed the procoagulant effects of PolyP 60 and PolyP 130 [129].

Despite the above-mentioned potential benefits, toxicity can also arise from PAMAM interactions with subcellar components or molecules. For instance, inhalation of a cationic PAMAM induced acute lung failure in mice through binding with ACE2 [122]. Collectively, these studies highlighting PAMAM/drug delivery system-induced molecular interactions at the level of gene expression or cellular proteins imply that such actions will have functional consequences which likely result in gross biological effects on cells and organs; and depending on the role of the target molecules impacted, these gross biological effects can be either beneficial or detrimental.

4.4. Interaction of PAMAMs with red blood cells and platelets

Since administration of PAMAM dendrimer delivery systems will likely include the parenteral route, their compatibility with blood components should be taken into consideration to ensure the safe application of these compounds. Following intravenous administration, the immediate gross toxicological effects observed with mainly cationic PAMAMs upon their interactions with blood components include red blood cell haemolysis, initiation of blood clot formation and disruption of key platelet functions [130,131].

In this context, many studies showed that cationic PAMAMs strongly interact with RBCs *in vitro*, causing significant haemolysis and inducing morphological changes resulting from the interaction between two oppositely charged membranes [132]. Further, the induction of haemolysis by cationic PAMAMs was time, concentration, and generation-dependent, as it increased gradually from G3 to G6. The onset of haemolysis was faster upon using cationic PAMAMs than anionic and neutral chemistries at similar concentrations and generations [132]. *In vivo* studies also revealed that cationic PAMAMs could trigger adverse blood clots in zebrafish embryos, resulting in blood toxicity [130]. While anionic and neutral PAMAMs caused a much less haemolytic effect; the role of the positively charged surface of PAMAMs in inducing haemolysis was confirmed as similar results were obtained when using a compound with similar amino surface chemistry and different polymeric structure [132].

In addition, cationic PAMAM dendrimers can trigger several changes in the morphology and the membrane integrity of RBCs (see Fig. 5)- which normally have the shape of a biconcave disc- in a generation-dependent manner [133,134]. The nature of these changes depended on the concentration of PAMAMs, i.e. a concentration of 1 nM induced echinocytic transformation, using 10 nM elongated the cells and created spindle-shaped forms, and finally, using 100 nM created drepanocyte-like cells [133,134]. It was also suggested that cationic PAMAMs could change the mobility and arrangement of fatty acid chains in the bilayer of RBCs cellular membrane, which might be linked to the observed morphological changes [133].

Regarding the impact of PAMAMs on platelets, it was reported that platelet morphology and function was altered by cationic PAMAMs, leading to increased aggregation [131,135]. In rodents, administering cationic PAMAMs intravenously produced a disseminated intravascular coagulation-like condition resulting in rapid

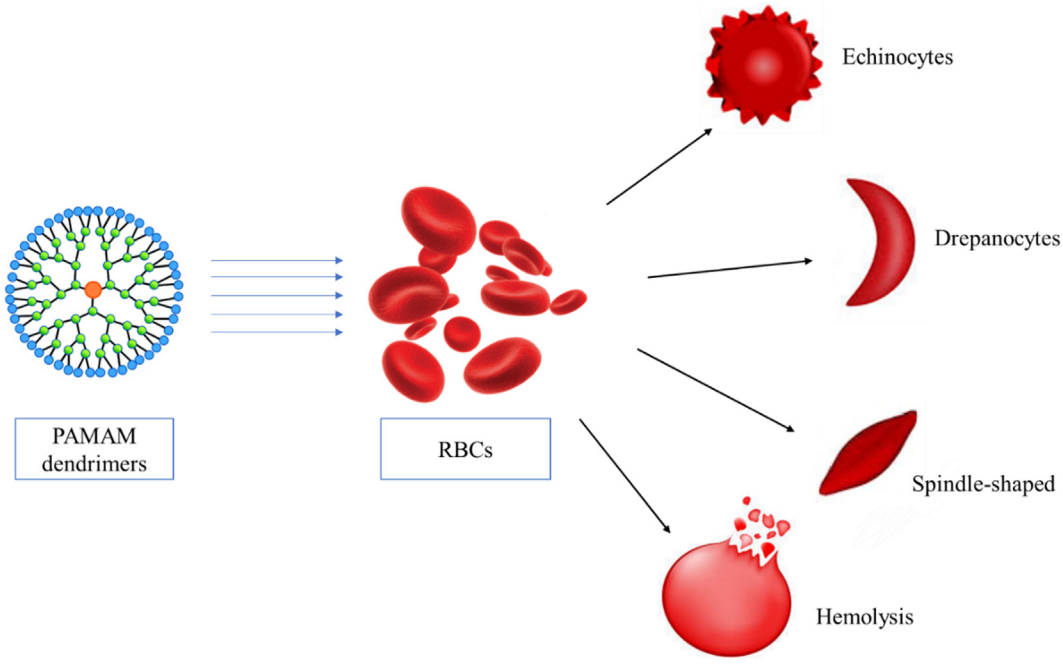

Fig. 5. Effect of PAMAMs on RBCs morphology. PAMAM dendrimers induce several morphological changes in RBCs, e.g., echinocytes transformation, drepanocytes transformation, spindle-shaped transformation [133–134], in addition to the incidence of hemolysis [132].

mortality [136]. Taken together, these data support that the trend of PAMAMs toxicity towards RBCs and platelets is surface chemistry, generation, and dose-dependent. Although cationic PAMAMs are usually the most bioactive, surprisingly, red blood cell antigen–antibody recognition was impaired after applying anionic G4.5 PAMAM, while similar concentrations of cationic and neutral PAMAMs did not cause a visible impact on the RBC agglutination. The reason for this was not entirely clear [137] but reflects the unique nature of biological actions of the different PAMAMs.

4.5. Cell toxicity and triggering cellular death via apoptosis, autophagy or necrosis

Like most polymeric delivery systems, PAMAM dendrimers also show some degree of cytotoxicity that is dependent on dose, time of exposure, generation, surface chemistry, and cell type. As a consensus, positively charged naked PAMAMs can exhibit significant cytotoxicity (assessed in terms of reduced cell viability or cell death) compared to anionic and neutral counterparts that are reported to minimally affect cell viability [59,120].

Cytotoxicity of PAMAMs has mainly been studied in cell lines *in vitro*. There is some criticism that use of cell lines may not be truly representative of the likely toxicity of PAMAMs *in vivo* and thus, primary cells may be a better model. Using primary aortic vascular smooth muscle cells, cell viability of different cationic PAMAM dendrimer generations (G4, G5, G6, G7) and surface chemistries (cationic, neutral and anionic) were compared. Cytotoxicity (reduced cell viability as measured by trypan-blue exclusion) was dose-dependent for cationic G5 and increased with increasing cationic PAMAM dendrimer generations. Generation matched neutral and anionic PAMAMs had little or no effect on cell viability at the doses studied [120]. Thus, PAMAM-mediated gross toxicity in primary cells is dependent on the physicochemical properties of the dendrimers- a fact that was also concluded from studies in different cell lines (see reviews by [59]. The cytotoxic nature of cationic polymers including PAMAMs has led to them being explored for anticancer applications *in vitro* and *in vivo* [47,49,50,52,54,138].

The toxicological/biological effects of PAMAMs *in vivo* are also largely mediated by cationic surface chemistry [37,59]. For example, a recent study examining the *in vivo* nanotoxicological effects of cationic G6 PAMAM in the heart, showed that chronic i.p. administration of this PAMAM in rats significantly impaired the recovery of cardiac function following ischemia–reperfusion injury [139]. Exposure of isolated hearts to cationic G6 PAMAM led to increased markers of cardiac damage [139] most likely indicating PAMAM-mediated myocardial injury and cell death.

Herein, we will discuss key studies investigating cellular death mechanisms induced by PAMAM dendrimers: namely autophagy, apoptosis, and necrosis (Fig. 6).

4.5.1. Autophagy

Autophagy (or autophagocytosis or "self-eating") is an intracellular degradation system wherein old or damaged organelles, unneeded proteins, or pathogenic agents, are sequestered in autophagosomal vesicles and digested by lysosomal enzymes with digestion products being released back into the cytoplasm- a process that can ultimately lead to cell death [140–142]. For the discovery of the molecular mechanisms of autophagy, a Nobel prize was awarded to Yoshinori Ohsumi in 2016 [143].

Cationic PAMAM dendrimers were proven to trigger autophagy in different cell lines. G3 cationic PAMAM specifically induced the accumulation of autophagosomes in human lung adenocarcinoma A549 cells through deregulating the Akt-TSC2-mTOR signaling pathway; this appeared to occur in the absence of apoptosis as the hallmarks of apoptosis (DNA fragmentation and Caspase-3 activity) were unaltered [124]. However, cationic G5 PAMAMs were reported to trigger apoptosis alongside autophagy in HepG2 hepatocellular carcinoma cells [144]. The hepatotoxicity of PAMAM dendrimers could be significantly reversed by inhibiting autophagy *in vivo* highlighting an important role of autophagy in PAMAM-induced liver injury [144]. CNS toxicity of PAMAM dendrimers in several glioma cell lines was also linked to induction of autophagy upon treatment with cationic G5 PAMAMs [145]. Different mechanisms induced by PAMAMs leading to autophagy were proposed, such as decreasing phosphorylation in Akt/mTOR

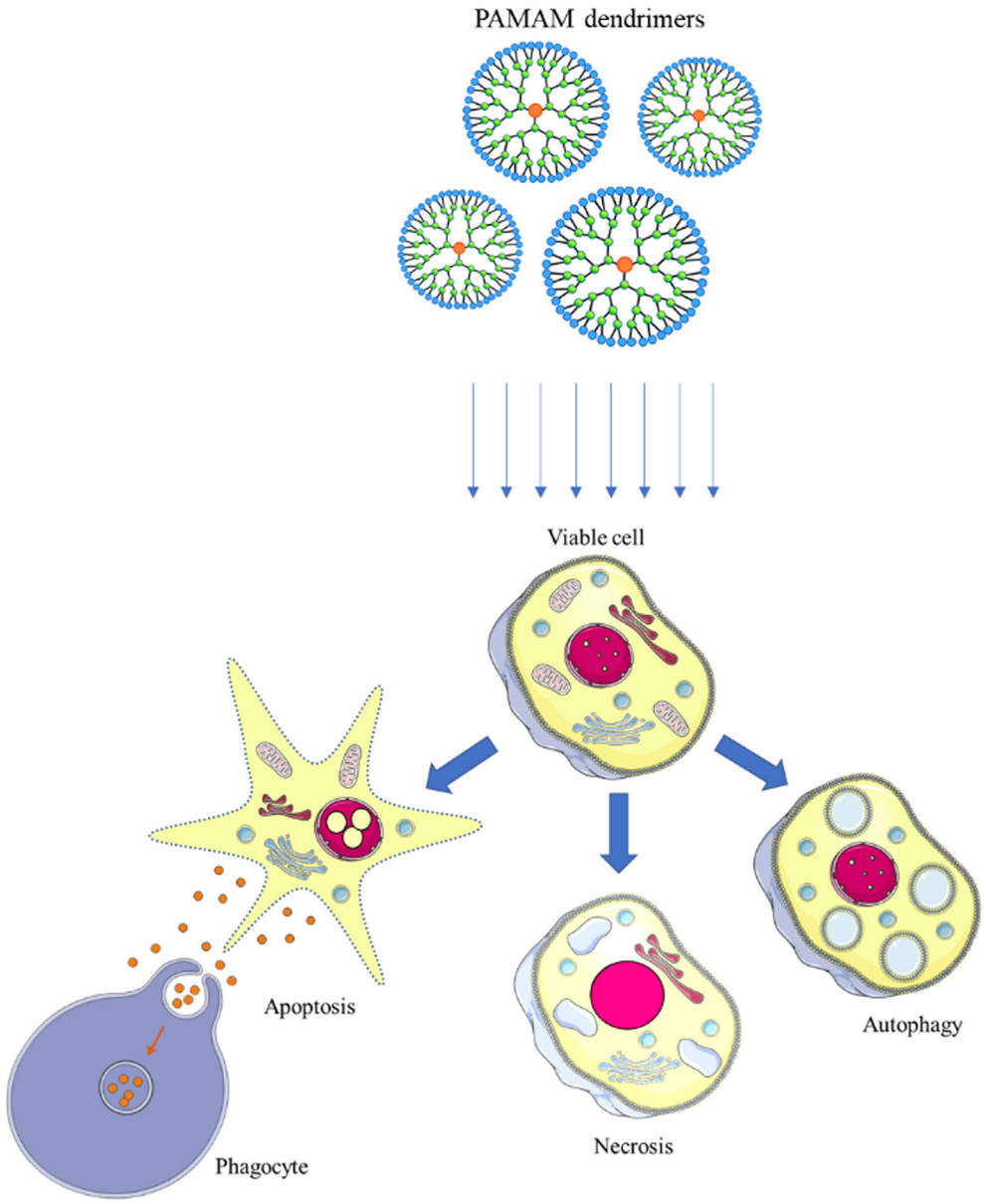

Fig. 6. Induction of cell death by PAMAM dendrimers. PAMAM dendrimers can trigger apoptosis, necrosis, and autophagy based on their molecular size/generation and surface chemistry (see main text for further explanantion).

signaling pathway and activating ERK1/2 signaling [124,144,145]. Also, an association between oxidative stress and autophagy triggered by cationic PAMAMs was established, as inhibiting ROS production reduced the observed toxicological effects [145–147]. The application of antioxidants such as N-acetyl cysteine, tocopherol and lipoic acid reduced PAMAM dendrimer-mediated autophagy and neuronal cell toxicity, thereby implying that using antioxidants and/or autophagy inhibitors could be a useful strategy to prevent PAMAM-mediated toxicity [147].

4.5.2. Apoptosis and necrosis

Apoptosis (also known as programmed cell death or "cell suicide") and necrosis (uncontrolled cell death) are the more widely reported methods by which cells can die [142]. Many studies have reported on the apoptosis and necrosis induced by PAMAM dendrimers, including the influence of different surface chemistries and generations. G4 cationic PAMAMs resulted in apoptotic effects with weak necrosis in different cell lines; human acute T-cell leukemia Jurkat cells and human lung cells, WI-26 VA4 [148,149]. Apoptosis in these cell lines was thought to be via disruption of mitochondrial membrane potential (MMP) and activation of ATM-mediated DNA damage [148,149]. Higher generations of cationic PAMAMs triggered both apoptosis and necrosis in KB cells and RAW 264.7 murine macrophage-like cells, with a difference in the mechanism of action being reported in each cell line [150,151] - again highlighting the influence of cell type in the biological actions of PAMAMs. In KB cells, cell death was mediated by lysosomal/mitochondrial pathways as PAMAMs were localized in the lysosomal compartment causing an increase in lysosomal pH and cytotoxicity [151]. On the other hand, both apoptosis and necrosis were reported in RAW 264.7 cells that could be inhibited by a caspase inhibitor, indicating that the mechanism is Caspase-dependent [150]. Recently we showed in breast cancer cells lines that cationic, but not anionic or neutral, PAMAMs can induce substantial cell apoptosis via up-regulation of apoptotic markers (Bax, Caspases-3, 8 and 9) and down-regulation of Bcl-2 [152].

In addition, two different structural architectures of G6 PAMAM dendrimers (PF and SF) triggered apoptosis in HEK 293 cells- indicating that irrespective of the branching structure, PAMAMs can commonly induce cell death via apoptosis [71]. Apoptosis in HEK293 cells as well as HaCaT cells, and fish hepatocellular carcinoma cells, appeared to be mediated by the generation of intracellular ROS [71,112,153]. Increased ROS production by PAMAMs has led to DNA damage, apoptosis, and even necrosis in a generation-dependent fashion (G4 < G5 < G6) [111,112,153]. Additionally, G7 cationic PAMAMs induced significant apoptosis in human umbilical vein endothelial cells (HUVEC) that was accompanied by necrosis at higher concentrations [154] -highlighting that the mechanism of cell death is markedly dependent on PAMAM concentration. These cellular effects of cationic PAMAMs even at low doses were accompanied by elevated expression of surface proinflammatory markers: ICAM-1 and phosphatidylserine- implying that PAMAMs can exert both pro-inflammatory and apoptotic effects in these endothelial cells. Interestingly, these authors also showed that G7 cationic PAMAM induced both plasma and mitochondrial membrane disintegration with the subsequent shedding of extracellular vesicles, including some of mitochondrial origin that were not associated with mitochondrial autophagy [154].

Taken together, these studies suggest that PAMAM-induced cytotoxicity can lead to cell death through the induction of multiple death mechanisms. As to which mechanism of cell death is initiated by exposure to PAMAMs is dependent on cell type, concentration/dose, time of exposure and the physicochemical properties of dendrimers (surface chemistry and generation/size/-molecular weight).

4.6. Embryotoxicity

Should PAMAMs reach the clinic, their potential use in pregnancy would need to be assessed. Indeed, several pre-clinical reports have suggested that PAMAM dendrimers can deliver siRNA and drug molecules during pregnancy for the treatment of different pathological conditions in the mother or the fetus [155–159]. Thus, it is important to understand the effects of PAMAMs on the development of embryos. It has now been reported that several different generations of cationic PAMAMs exhibited substantial toxicity to the embryo in different models. For instance, cationic PAMAMs induced apoptosis and attenuated the development of zebrafish embryos in a concentration-dependent manner [160,161]. Additionally, toxic effects triggered by cationic PAMAM dendrimers were reported at the early stages of embryogenesis in chicken embryos. These effects were mediated by inhibiting angiogenesis as well as altering the expression patterns of many genes responsible for apoptosis and survival in the embryos, such as: ATF-3, Bcl-2, Caspase-8, FOXA-2, INHIBA, MAPRE2, RIPK-1, SERPINA-4, and VEGFC genes [162]. In contrast, PAMAMs with anionic or neutral surface charge/chemistry did not exhibit significant embryotoxicity, suggesting the greater safety of these compounds during pregnancy in comparison with cationic PAMAMs [154,162,163]. Thus, these data imply that use of cationic PAMAMs will likely be contraindicated in pregnancy.

4.7. PAMAMs as glucose scavengers/sequestrants

Polymeric sequestrants in the form of hydrogels and resins have been widely used to remove toxins, ions, bile acids, and fats, from the body (for review see [41,42,44]. For example, Kayexalate (sodium polystyrene) that sequesters excess potassium ions in the gut has been approved for the treatment of hyperkalemia since 1975 and Renagel, whose structure is based on polyallylamine, can bind to and lower serum phosphates in patients with chronic kidney disease (for reviews see [41,42,44].

There is now emerging evidence in the literature that PAMAMs can act as sequestrants/scavengers of glucose in several experimental models- a finding that potentially could be beneficial in treating diabetes and its complications. This glucose scavenging effect is mediated mainly by cationic PAMAMs and likely occurs via an interaction between PAMAMs terminal amino groups and glucose molecules to form N-glycosylated PAMAMs [164]. In particular, G4 cationic PAMAMs showed remarkable scavenging effects of excessive glucose in diabetic rats and inhibited long-term markers of diabetic hyperglycemia [165,166]. However, this was accompanied by much higher reported incidences of death in treated animals than in controls [165]. Subsequently, it was reported that G2 and G4 cationic PAMAMs could prevent non-enzymatic modifications of biomacromolecules by acting as anti-glycation agents. Nonetheless, no difference in efficacy was noted between G2 and G4 PAMAMs in terms of scavenging glucose and hindering protein glycation, although the structure of G2 allows better glycation than G4 [167]. Another study highlighted that among different (G2, G3, and G4) PAMAMs, G3 showed the most significant reduction in markers of severe hyperglycemia, implying that higher generations of PAMAM dendrimers might result in less glucose scavenging [164]. Additionally, beneficial effects in lowering blood glucose by G5 and G6 cationic PAMAMs were reported in diabetic rats, with a greater effect seen in the lower generations, consistent with previous studies [164]. These differences between PAMAM generations might be due to the significant rigidity of the structure of higher generations, making it more difficult to capture or entrap glucose molecules.

Interestingly, PAMAMs with anionic surface chemistry (G5.5) also showed a modest but significant effect in lowering blood glucose, which might encourage employing these PAMAMs over their cationic counterparts since they are generally less toxic [37]. Chronic daily i.p. administration of cationic PAMAM for one month did not appreciably reduce blood glucose, implying that much longer administrations of around 2-months might be needed to observe significant glucose lowering in animals with cationic PAMAMs [37]. However, the precise mechanism by which the anionic PAMAM dendrimer achieved glucose lowering was not studied and remains to be elucidated.

Though not entirely clear, taken together these studies imply that the possible mechanisms by which PAMAMs sequester glucose rely on the open structure of lower generations (up to G4 or G5) to allow the relatively small sized glucose to entrap within the interior cavities of the dendrimer structure. In contrast, the more densely packed nanostructures resulting from the greater number of surface groups of the higher dendrimer generations might sterically hinder glucose entrapment into the inner, and likely smaller cavities. Alternatively, there is also evidence that non-enzymatic covalent attachment of the glucose molecules onto the amino surface groups can result in the formation of N-glycosylated (“glycated”) PAMAM dendrimers. Furthermore, the ability of PAMAMs to sequester glucose appears to be dependent on dendrimer generation, dose/time of exposure and on surface chemistry.

4.8. PAMAMs as modulators of the inflammatory responses

Surprisingly, PAMAM dendrimers can elicit opposing effects on inflammation (see Table 2 for a summary of the key studies). It was demonstrated that PAMAMs produce an anti-inflammatory effect, which makes them drug candidates for the treatment of several inflammatory conditions [168]. However, other studies revealed that some PAMAMs (even the same PAMAMs that have anti-inflammatory effects) induce inflammatory responses and secretion of pro-inflammatory cytokines in non-diseased animal models [168].

Table 2
Effects of PAMAM dendrimers on inflammatory responses.

PAMAM surface chemistry	Generation	Model	In-vivo test	In-vitro test	Reported effect	Reference
Cationic	0	Female CD-1 mice	Murine air pouch modelm	Detection of cytokines/chemokines	Pro-inflammatory	Durocher and Girard [172]
	1					
	2					
	3	Female CD-1 mice	Murine air pouch modelm	Detection of cytokines/chemokines	Pro-inflammatory	Durocher and Girard [172]
		THP-1 and U937 cells	NA	RT-PCR Electrophoretic mobility shift assay Cytokine assay	Pro-inflammatory	Janaszewska et al [171]
	4	HaCaT cells		ROS measurement TNF-α and IL8 expression	Pro-inflammatory	Mukherjee et al [153]
		J774A.1 macrophage cell	NA	ROS measurement Cytokine assay	Pro-inflammatory	Naha et al [111]
		THP-1 and U937 cells	NA	RT-PCR Electrophoretic mobility shift assay Cytokine assay	Pro-inflammatory	Janaszewska et al [171]
		Male albino rats	Carrageenan-induced edema Cotton pellet test Adjuvant-induced arthritis	Macrophage assays Measurement of nitrite concentrations Cyclooxygenase assay	Anti-inflammatory	Chauhan et al [169]
	5	HaCaT cells	NA	ROS measurement TNF-α and IL8 expression	Pro-inflammatory	Mukherjee et al [153]
		J774A.1 macrophage cell	NA	ROS measurement Cytokine assay	Pro-inflammatory	Naha et al [111]
		Male Sprague-Dawley rats	Intratracheal instillation	Evaluation of BAL fluid Detecting PGE2 metabolites	None	Wangpradit et al [173]
	6	HaCaT cells	NA	ROS measurement TNF-α and IL8 expression	Pro-inflammatory	Mukherjee et al [153]
		J774A.1 macrophage cell	NA	ROS measurement Cytokine assay	Pro-inflammatory	Naha et al [111]
Anionic	4.5	Male albino rats	Carrageenan-induced edema Cotton pellet test Adjuvant-induced arthritis	Macrophage assays Measurement of nitrite concentrations Cyclooxygenase assay	Anti-inflammatory	Chauhan et al [169]
		Mice	Caerulein-induced acute pancreatitis	Hematoxylin-eosin and Immunohistological Staining to Pancreatic Tissues Macrophage infiltration in pancreas	Anti-inflammatory	Tang et al [170]
			LPS-stimulated mouse peritoneal macrophages	RT-PCR and Western Blot NF-κB nuclear translocation		
Neutral	5	Mice	Caerulein-induced acute pancreatitis	Hematoxylin-eosin and Immunohistological Staining to Pancreatic Tissues Macrophage infiltration in pancreas	Anti-inflammatory	Tang et al [170]
			LPS-stimulated mouse peritoneal macrophages	RT-PCR and Western Blot NF-κB nuclear translocation		

4.8.1. Anti-inflammatory effects mediated by PAMAMs

The anti-inflammatory effects of naked PAMAMs were accidentally discovered in male albino rats as demonstrated by three *in vivo* models/assays: the carrageenan-induced paw edema model, the cotton pellet test, and the adjuvant-induced arthritis assay [169]. This study found that G4 cationic PAMAMs exhibited more significant anti-inflammatory effects compared to G4.5 anionic PAMAMs in a dose-dependent manner, implying an important influence of dendrimer surface chemistry. Moreover, PAMAM dendrimers exhibited synergy with the anti-inflammatory effects of indomethacin [169]. G4.5 anionic and G5 neutral PAMAM dendrimers afforded significant protection from pancreas injury in a cerulean-induced acute pancreatitis mouse model, by reducing the ensuing inflammatory responses [170]. In addition, PAMAMs dysregulated gene expression of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and TGFβR-1, in pancreatic tissues *in vitro* and in mouse peritoneal macrophages *in vivo* [170]. Another proposed mechanism behind PAMAMs anti-inflammatory actions was inhibition of NF-κB in macrophages [170]. The total count of plasma white blood cells and monocytes was also reduced significantly upon treatment with PAMAMs *in vivo*. Further, the protective effects of anionic PAMAMs in developing acute pancreatitis *in vivo* were more significant than neutral PAMAMs [170]. Combined with previous findings, it can be stated that the anti-inflammatory effects of PAMAM dendrimers are most pronounced with cationic surface chemistry and least likely with neutral chemistry (i.e. cationic > anionic > neutral).

4.8.2. Pro-inflammatory effects mediated by PAMAMs

In order to investigate the pro-inflammatory activity of PAMAM dendrimers, G3, G4, G5, and G6 cationic PAMAMs were tested *in vitro* in THP-1, U937, and mouse J774A.1 macrophage cell lines [111,171]. It was found that PAMAMs, in a concentration-, time- and generation-dependent manner, increased intracellular ROS production. In addition, cytokine assays showed an increase in the pro-inflammatory mediators IL-6, TNF-α, and MIP-2 [111]. Moreover, PAMAM dendrimers contributed to the inflammation by activating signal transduction pathways related to NF-κB and thus, increased the expression of NFKBIA, BTG2, IL1 β, and TNFα [171]. Another *in vivo* study in female CD-1 mice revealed similar results using a murine air pouch model, as G0, G1, G2, and G3 cationic PAMAMs exhibited major pro-inflammatory actions [172]. PAMAM-induced inflammation was indicated by increased leukocytes migration into air pouches, mainly neutrophils, accompanied by an increase in the local production of several cytokines/chemokines in a generation-dependent manner [172].

Despite these reports, another study evaluating the impact of G5 cationic PAMAMs *in vivo* in male Sprague-Dawley rats reported no inflammatory responses. Cationic PAMAMs did not elevate the pro-inflammatory cytokines (IL-1β and TNFα) as detected by a bio bead-based ELISA assay [173]. The difference in the reported behaviour of similar types of PAMAM dendrimers in modulating inflammatory responses is probably due to using different models, different concentrations, and different routes of administration. Moreover, the pro-inflammatory effect was mainly found in normal, non-diseased animal models rather than in animals bearing a disease [168]. These contradictory data show the need for a more in-depth investigation of PAMAMs inflammatory responses in different models before drawing a conclusion related to these effects.

4.9. Antimicrobial effects: Can PAMAMs serve as novel antimicrobial agents?

The emergence of antibiotic resistance and the limited development of novel antimicrobial agents has led to the search for newer antimicrobial nanomaterials as well as nanotechnology solutions to overcome the problem of drug resistance to existing therapies. Several polymeric nanoparticles, including PAMAMs, have been considered for potential use as antimicrobial agents with known activity against many bacteria, viruses, fungi, and protozoa, (for a recent review see [174]) either because of their intrinsic antimicrobial actions including modulation of mechanisms leading to antimicrobial drug resistance or through acting as carriers for the delivery of well-established antimicrobial agents such as antibiotics or antiviral agents [49,174]. Herein, we focus our discussion on the antimicrobial activity of naked PAMAM nanoparticles and not PAMAMs conjugated to antimicrobial agents, for which the reader is referred to other recent reviews [49,174].

Although the precise mechanisms of antimicrobial action remain poorly understood, it is generally thought that polymeric nanoparticles can penetrate and disrupt the microbial cell membrane integrity, induce ROS production, interact/interfere with DNA/RNA, proteins or enzyme systems and efflux transporters like p-glycoprotein, as well as inhibit biofilm formation [49,174]. In general, the antimicrobial activity of polymeric nanoparticles appears to be dependent on their physicochemical properties including surface chemistry/charge, size and shape, surface-to-volume ratio, and zeta potential [49,174].

Over recent years, as a result of their innate biological actions, PAMAM dendrimers/nanoparticles have been widely investigated for their antimicrobial effects, particularly cationic PAMAMs, which might have a promising future in this area [174–175] (see also Table 3). The antimicrobial properties of PAMAM dendrimers were explored *in vitro* in a wide variety of Gram-negative pathogens incuding *E. Coli, P. Aeruginosa, A. baumannii, S. typhimurium, S. dysenteriae, K. pneumoniae,* and *P. mirabilis*, as well as in Gram-positive bacteria such as *S. aureus, B. subtilis,* and *MRSA* [155,175–179]. These bacteria, because of their multidrug-resistant strains, represent a global challenge in terms of treatment [180,181]. PAMAM dendrimers exhibited substantial dose-dependent antimicrobial effects, reported in many investigations, with a generally greater effect against standard strains compared to strains isolated from clinical samples [175,178]. Although some conflicting reports exist [176], Gram-positive bacteria were, in general, more sensitive to PAMAMs treatment than Gram-negative ones, mainly due to their differences in the bacterial cell wall structure, with some exceptions such as *Proteus mirabilis* and *Salmonella typhimurium*. On the other hand, the least sensitive bacteria to PAMAMs appeared to be *Acinetobacter baumannii* [175,177,178]. The fact that PAMAMs are also effective against a fungus, *A. niger* and a yeast strain, *Candia albicans* [177] means that PAMAM dendrimer-mediated antimicrobial activity extends beyond bacteria, and as such these dendrimer nanoparticles might be considered as novel broad spectrum antimicrobial agents.

PAMAM dendrimers also showed a surface chemistry dependency in regard to their antimicrobial effects, as these were greater with cationic PAMAMs relative to their anionic or neutral counterparts [155,179]. This finding was supported by surface PEG-coating of PAMAM dendrimers that not only decreased the surface charge but also diminished their antimicrobial effects [176,182]. However, neutral PAMAMs also showed good activity against *Escherichia coli*-induced ascending uterine infection *in vivo* in a guinea pig model of chorioamnionitis and as these also exhibited less toxicity to normal cells, neutral PAMAMs might be favoured over cationic PAMAMs which generally have higher toxicity in normal cells [155]. The role of dendrimer generation in mediating the antimicrobial effects of PAMAMs was also reported with antimicrobial activity reportedly higher with increasing generation [179]. This is most likely due to the highly branched structure, large molecular weight, and the high number of terminal amino groups/positive charges present in the higher generations of cationic PAMAMs [178]. However, in another study no difference in antimicrobial activity against Pseu-

Table 3
Antimicrobial effects of PAMAM dendrimers.

PAMAMs used		G2			G3/3.5			G4			G5	G6			G7				
		NH2			NH2	COOH		NH2		OH	NH2	NH2			NH2				
Reference		[177]		[179]	[179]	[179]	[155]	[179]	[155]	[155]	[179]	[175]			[178]				
Origin of bacteria parameter		Standard MIC (ppm)	MBC (ppm)	MIC50 (μg/mL)	MIC50 (μg/mL)	MIC50 (μg/mL)	Isolated IC50 (mg/mL)	MIC50 (μg/mL)	Isolated IC50 (μg/mL)	IC50 (mg/mL)	MIC50 (μg/mL)	Isolated EC50 (μg/mL)	Standard MIC (μg/mL)	MBC (μg/mL)	Isolated MIC (μg/mL)	MBC (μg/mL)	Isolated MIC50 (μg/mL)	MIC90 (μg/mL)	MBC50 (μg/mL)	MBC90 (μg/mL)
Gram-negative bacteria	E. coli	2	2.6	–	4.931	> 1000	22	–	3.8	5.4	–	–	2.5	100	25	200	4	8	256	256
	P. aeruginosa	2	3.3	–	–	–	–	–	–	–	–	1.5	2.5	100	2.5	100	4	8	256	256
	A. baumannii	–	–	–	–	–	–	–	–	–	–	–	2.5	200	25	200	4	8	128	256
	S. typhimurium	–	–	–	–	–	–	–	–	–	–	–	0.025	2.5	0.25	25	–	–	–	–
	S. dysenteriae	–	–	–	–	–	–	–	–	–	–	–	0.25	100	0.25	50	2	2	64	128
	K. pneumoniae	–	–	–	–	–	–	–	–	–	–	–	0.25	100	0.25	100	2	4	128	256
	P. mirabilis	–	–	–	–	–	–	–	–	–	–	–	0.25	100	0.25	100	2	2	128	128
Gram-positive bacteria	S. aureus	5.2	15.6	26.77	9.374	> 250	–	5.962	–	–	2.881	20.8	0.25	100	0.25	100	4	8	256	256
	B. subtilis	3.9	3.9	–	–	–	–	–	–	–	–	–	0.25	50	0.25	50	4	4	128	128
	MRSA	3.9	3.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fungus	A. niger	7.8	15.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Yeast	Candia albicans	7.8	62.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

domonas aeruginosa and Staphylococcus aureus was observed between G5 and G3 cationic PAMAMs [182] - implying that the type of micro-organism studied may also be an important determinant of PAMAM antibacterial activity.

Regarding the mechanisms behind cationic PAMAMs antimicrobial effects, it was proposed that the electrostatic interaction between the positively charged surface of PAMAMs and the negatively charged bacterial cell wall causes disruption and disintegration of the lipid bilayer with subsequent release of intracellular contents such as electrolytes and nucleic acids leading to death of the microorganism [155,175,178,179]. For the other types of PAMAMs, it was found that neutral PAMAMs can change the permeability of the bacterial cellular membrane, while anionic PAMAMs can also damage the membrane causing bacterial lysis [155].

Collectively, these studies suggest that, consistent with their toxicity profile in mammalian cells *in vitro* and *in vivo* [120], PAMAM dendrimers, especially cationic, and of higher generations (>G3, as lower generations do not exhibit appreciable activity) exhibit innate antimicrobial activity that may be clinically useful. PAMAMs, similar to other cationic polymers/nanoparticles, can exert their antimicrobial actions via binding to and disrupting the integrity of negatively charged microbial membranes to induce leakage of cell contents. Given the same mechanisms are likely responsible for the cellular toxicity of higher generation PAMAMs in host cells, topical infections such as those on the skin or vaginal mucosa, might be more amenable targets (low-hanging fruit) for PAMAM-based antimicrobial therapies that are likely to have limited systemic side-effects. However, mitigation of some of the host cell toxicities associated with PAMAMs by strategies such as PEGylation (see also section below), might broaden their application to systemic infections- though a balance in the degree of charge neutralization will need to be sought to maximise microbial toxicity and minimise host cell toxicity. Such an approach may also facilitate the systemic administration of PAMAMs as vectors for the delivery of conventional antimicrobial agents where significant synergistic effects of dendrimer with drug have been noted [49,174].

5. Potential strategies to reduce PAMAM cell toxicity

Despite all their potential therapeutical benefits, the clinical utilization of PAMAM dendrimers faces a major obstacle, which is the significant non-specific cytotoxicity seen with cationic PAMAMs in particular. In fact, the toxicity of cationic polymers and nanomaterials is common in delivery systems, mainly due to the interaction between their dense surface positive charge and the negatively charged cellular membrane, causing cell lysis and death via apoptosis, necrosis and/or autophagy [125,183,184]. Nevertheless, several strategies (see Table 4) have been proposed to diminish this toxicity, such as PEGylation, i.e., covalent linking to polyethylene glycol (PEG). Many studies stated that conjugating PEG chains with cationic polymers decreases their intrinsic toxicity, enhances their biocompatibility, and increases their half-life [15,185,186]. The conjugation of other surface molecules was found to also diminish the toxicity of PAMAMs through masking their cationic surface charge to a certain extent, like acetyl group, lauroyl chloride, 4-carbomethoxypyrrolidone, and many other molecules [187–190]. On the other hand, other strategies have been proposed in the literature to manage the cytotoxicity of cationic PAMAMs while preserving their cationic surface, such as optimizing the route of administration. For instance, it was found that intra-tracheal administration can avoid inflammatory responses caused by cationic PAMAM dendrimers in rats [173]. Furthermore, intraperitoneal administration of PAMAM dendrimers in rats was reported to be

Table 4
The most common strategies to reduce PAMAM-mediated toxicity.

Method	Description	Reference
Surface modification	PEGylation	[15,205,185–187]
	Acetylation	[189]
	Lauroyl chloride conjugation	[187–188]
	4-carbomethoxypyrrolidone conjugation	[190,206]
	Carboxymethylchitosan conjugation	[207]
	N-(2-hydroxydodecyl) conjugation	[208]
	Histidine conjugation	[209]
	Pyrrolidine modification	[171,210]
Route and targeted delivery	Intra-tracheal administration	[173]
	Intraperitoneal administration	[37,194]
	Targeted delivery (e.g. antibody conjugation)	[191–193]

favoured over the intravenous route where haemolysis can occur [37]. In contrast to the rapid exposure of dendrimer to blood components via the i.v route, cationic PAMAMs administered by the i.p. route are thought to be slowly released from the peritoneum into the vascular system resulting in minimal haemolysis. Moreover, targeted delivery of PAMAM dendrimers to selected cells may allow localization to specific tissues/organs and thereby minimizing systemic toxicity. For example, grafting the anti-HER2 antibody, trastuzumab, onto the surface of PAMAMs allows selectivity and specificity towards HER2-positive breast cancer cells rather than other cells [191–193] (Table 4). The development and optimization of such toxicity-reducing strategies could broaden the horizon of clinical applications possible for PAMAMs, especially cationic surface chemistries, by facilitating the exploitation of their many innate biological activities.

6. Concluding remarks and perspectives

It is becoming increasingly apparent that PAMAM dendrimer nanoparticles exhibit innate or intrinsic biological properties beyond their ability to improve drug delivery as has been highlighted in this review (for a summary see Fig. 7). These include modulation of gene expression, interaction with protein molecules and modulation of cell signaling cascades including immune responses that ultimately manifest in multiple gross biological changes including haemolysis and cell death. The nature and extent of biological effects are heavily dependent on the physicochemical properties of PAMAMs. In almost all cases, in addition to the usual dose and time dependency, the biological effects of PAMAMs were dependent on the dendrimer generation, surface charge/chemistry, structural architecture and the cell or biological system studied.

Some of the biological effects might be considered detrimental and may actually be regarded as toxicological effects. There is the obvious concern of the general cytotoxicity of PAMAMs, mainly with a cationic surface charge, that can result in reduced cell viability and death. However, these toxicities may be mitigated by careful selection of the PAMAM with the right physicochemical properties (e.g. anionic or neutral surface chemistries) or through, amongst others, PEGylation. In addition, there is the risk of haemolysis when PAMAMs are administered parenterally into the circulation. Of note here is that despite a significant body of evidence documenting haemolysis with cationic PAMAMs, this is largely based on in-vitro (test-tube) assays and/or following rapid direct exposure of PAMAMs into the bloodstream upon intravenous administration (e.g. [130,133]. In contrast, cationic PAMAMs administered by intraperitoneal injection, presumably because PAMAMs first enter the peritoneum before slowly diffusing into the bloodstream, cause minimal haemolysis with little or no haematological toxicity being reported with neutral or anionic surface chemistries in animals [37,194]. Thus, cationic PAMAM-mediated effects on haemolysis can be circumvented through a careful selection of the route of administration and therefore may not prevent scientists exploiting the many biological properties of PAMAMs for potential clinical applications.

Several of the biological properties of PAMAMs, as reviewed herein, may have the potential for clinical benefit. There is the obvious potential for exploiting the cell toxicity of cationic PAMAMs for use as anti-cancer agents or, more appealingly, as antimicrobial agents (see Section above). However, this might necessitate surface charge modifications to balance or optimise the host-vs-target cell toxicity profile of the cationic PAMAMs.

Of particular interest is the potential exploitation of several biological actions reported for PAMAMs (reviewed above) that might be clinically useful in the treatment of diabetes and its complications. Diabetes mellitus (DM) is a major debilitating disease characterized by hyperglycaemia whose incidence is progressively increasing globally; currently over 463 million adults have diabetes with the International Diabetes Federation predicting over 700 million sufferers by 2045 [195]. It is the complications of diabetes such as cardiovascular, renal, and retinal dysfunction that leads to increased patient morbidity and mortality. Of these, cardiovascular complications that can result in stroke and cardiomyopathies are 3- to 5-fold more likely in diabetes (for a recent review see [114]. Although several anti-diabetic therapies are available, these may not be effective in all patients and despite therapy, many sufferers go on to develop the complications of the disease which are often more devasting than the disease itself [114]. So, could the innate biological properties of PAMAMs be exploited therapeutically for the potential treatment of diabetes and/or its complications?

Several reported biological actions of PAMAMs support their use in diabetes management. **Firstly**, PAMAMs appear to have the ability to sequester glucose and at least the lower generations appear to be useful glucose-scavenging agents. As a result of reducing blood glucose levels, they can also reduce the nonenzymatic modifications of macromolecules that are formed because of hypergylcemia and are known to lead to complications associated with diabetes [114,164,196].

Secondly, PAMAM-mediated interactions with Amylin (or human islet amyloid polypeptide (hIAPP)) can prevent its aggregation and deposition as amyloid deposits thereby preventing beta-cell toxicity in the islet of Langherhans [126]. Amylin is normally released together with insulin from pancreatic beta-cells to regulate glucose levels, but its deposition as amyloid plaques can be damaging to beta-cells in the pancreas. Impaired pancreatic beta-cell function is associated with both Type 1 and Type 2 DM [114]. Since PAMAMs are reported to preferentially accumulate within the pancreas [78], this biological activity might represent a viable therapeutic option through passive targeting of PAMAMs to this important organ.

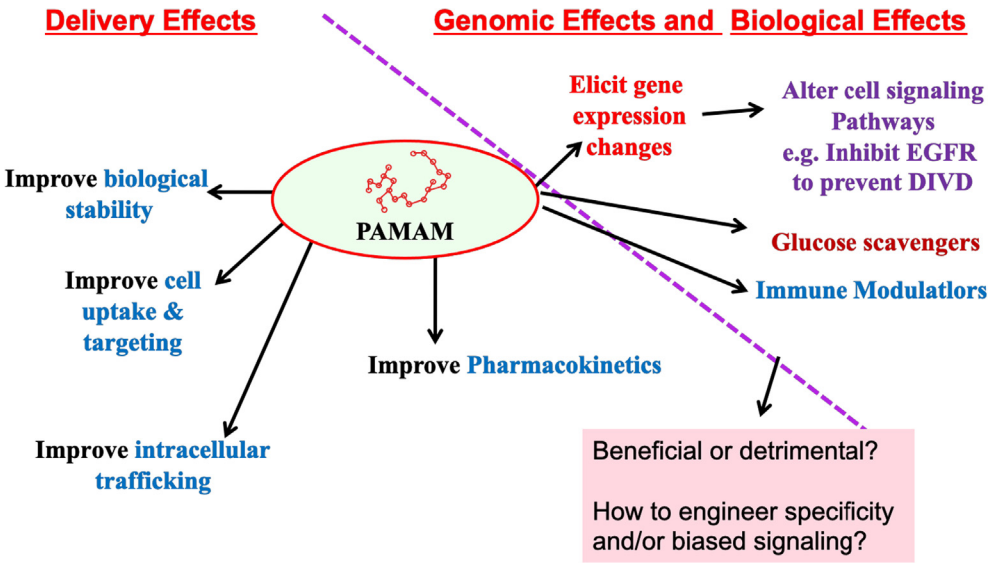

Fig. 7. The biological properties of PAMAMs beyond drug delivery. In addition to having drug delivery enhancing properties such as improving biological stability, cell uptake and trafficking or pharmacokinetics/pharmacodynamics of drugs, PAMAMs also exert multiple biological actions even in the absence of a drug pay-load. These intrinsic actions can occur at the genomic level (e.g. modulation of gene expression) or at the proteome level in modulation of protein interactions or cell signaling cascades. The example given is that of PAMAM-mediated EGFR inhibition which can prevent diabetes-induced vascular dysfunction (DIVD). They also have immunomodulatory and glucose sequestering ability. The biological actions of PAMAMs can be beneficial or detrimental; however, a key challenge for drug delivery and polymer scientists is to engineer specificity including biased signaling towards the desired biological or clinically beneficial properties.

Thirdly, PAMAMs can interfere with cell signaling pathways mediated via EGFR/ErbB family of receptor tyrosine kinases that are known to be involved in the development of cardiovascular complications of diabetes [37,114,70–73]. PAMAMs can correct diabetes-induced vascular dysfunction by blocking the actions of EGFR/ErbB2 receptors in animal models of diabetes [37,118] and might represent a new class of EGFR/ErbB receptor antagonists [37]. Since EGFR/ErbB receptors are also known to be involved in the development of other diabetes complications such as cardiac, renal, ocular and neuronal dysfunction [114], PAMAM use may also be important in preventing these other EGFR/ErbB driven complications of diabetes. The fact that negatively-charged, anionic PAMAMs are also effective as EGFR/ErbB blockers and can prevent diabetes-induced vascular complications [37] is appealing as they have a better toxicity profile than cationic PAMAM dendrimers.

Fourthly, since vascular complications of diabetes can result in poor wound healing and infections especially in diabetic foot ulcers, the antimicrobial actions of PAMAMs along with their intrinsic vasculoprotective actions could be combined for the potential treatment of diabetic wounds. Potentially topical formulations containing PAMAMs would be desirable to promote anti-infective activity, recovery of vascular function and promote wound healing in diabetes patients. The benefits of any topical formulation may be further enhanced by including PAMAMs that have been conjugated with conventional antimicrobial agents that are known to produce synergistic antimicrobial activity.

A potentially negative impact of PAMAMs on their use in diabetes could be envisaged through the possibility that a G4 PAMAM induces gene expression of dipeptidyl peptidase-4 (DPP4) [67] - (see also Table 1)- though this biological action is very speculative and based only on non-validated RNA seq data on gene expression in primary bronchial epithelial cells. DPP4 is the enzyme responsible for the rapid N-terminal cleavage and inactivation of glucagon-like peptide 1 receptor (GLP-1), a gut hormone produced by enteroendocrine L cells that enhances insulin release in response to ingested glucose [197–201]. Its up-regulation, if reproduced and shown to occur in the relevant tissues/cells, would compromise blood glucose lowering actions of GLP-1. Indeed, orally-acting DPP4 inhibitors such as Saxagliptin, and Sitagliptin that increase endogenous GLP-1 levels through blocking its degradation are available clinically. Thus, if increased DPP4 gene expression was truly problematic with PAMAM use, one could consider co-administration of DPP4-inhibitors to mitigate any consequences of raised DDP4 levels induced by the dendrimers. Another potentially more important concern is that chronic vivo administration of PAMAMs can attenuate the recovery of hearts from ischemia-reperfusion injury [139], though this adverse effect might also be easily mitigated by the co-administration of another agent, in this case, epidermal growth factor, an EGFR ligand that is known to be cardioprotective in myocardial I/R injury (for review see [114]).

From the many studies reviewed herein, it is likely that PAMAMs will have pleiotropic biological actions *in vivo* that may sometimes oppose or contradict the desired biological/clinical outcomes. Although these actions are a mix of broad or generalised biological effects (e.g. apoptosis) and organ- or tissue-specific (as in the case of disruption of amylin deposits in the pancreas), it is quite possible, due to the wide biodistribution of PAMAMs, that multiple actions could be occurring simultaneously *in vivo*. Indeed, the full range of biological properties of the different PAMAMs will become more apparent as further research is conducted on other potentially novel biological actions of PAMAMs *in vivo*.

Although the exact cellular consequences and interplay of the many intrinsic biological activities of these delivery systems in the body are likely to be very complex, their pharmacological and/or toxicological consequences will need to be fully characterized and understood prior to considering PAMAMs for a particular clinical application in nanomedicine. The challenge for polymer scientists will be to better design or engineer PAMAM constructs whose actions can be more defined or biased towards the specified or desired clinical effect including at the level of biased cell signalling (see Fig. 7). In addition, the costs of PAMAM-based therapeutics will need to be weighed against currently available standard of care therapies- though costs of manufacture will undoubtedly decrease with increasing demand as well as with advances in manufacturing processes.

An emerging theme from the studies reviewed herein is that the physicochemical properties of PAMAM dendrimer nanoparticles including size/generation, shape/structural architecture, rigidity/elasticity and surface charge/chemistry directly govern their interactions with cell membranes and biological barriers to influence their pharmacokinetics and bioavailability, as well as their ability to interact with blood components and cellular and subcellular components including gene-expression machinery and cell signaling protein networks. Although studies, mostly *in vitro*, have looked at each of these biological interactions independently and correlated them back to the physiochemical properties of PAMAMs, it remains to be elucidated as to how these interactions will collectively interplay and cross-talk in the more complex and dynamic *in vivo* situation. Where, for example, additional biological environment-dictated modifications of the nanoparticle surface (e.g. acquisition of a protein corona [202,203]) will likely occur and thus, alter the baseline surface properties of PAMAM nanoparticles further in a more dynamic fashion. Though it is reported that these surface-protein interactions can also be reduced by making the PAMAM surface more neutral [204], it is still likely that PAMAM nanoparticles with differing extents of bound corona proteins will represent an heterogenous mix *in vivo* in which every nanoparticle could potentially behave as (or be seen by the cellular machinery as) a different biological entity. The precise collective impact of all these differing physiochemical variables for a given PAMAM nanoparticle-mediated biological or clinical effect in intact animals or in humans, including as a function of time and dose, requires further study. However, in other nanoparticle systems, it has been suggested that through a greater understanding of the relationship between physicochemical properties and biological outcomes, and by exquisite control of nanoparticle size, shape and architecture, as well as through careful selection of cell or tissue-specific targeting moieties and inclusion of biologically responsive groups, nanoparticle delivery systems can now be produced with smart/intelligent designs so as to tailor delivery to a particular application (e.g. [1,2]). Lessons from the use of other nanoparticles as precision medicines might provide hope for the tailored use of PAMAMs in the clinic.

In conclusion, from the vast array of biological properties reviewed here, and through careful design or selection of PAMAMs with the desired physicochemical properties, there is significant scope for the potential clinical application of PAMAMs especially as antimicrobial agents and/or for the potential treatment of diabetes and its complications. However, greater research is needed to fully understand the true implications of the biological and toxicological properties of PAMAMs in the potential clinical development of these important class of dendrimers as drug delivery systems and as pharmacologically active agents in their own right.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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