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Pluronic® block copolymers: novel functional molecules for gene therapy

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Abstract

Pluronic® block copolymers are recognized pharmaceutical excipients listed in the US and British Pharmacopoeia. They have been used extensively in a variety of pharmaceutical formulations including delivery of low molecular mass drugs and polypeptides. This review describes novel applications of Pluronic® block copolymers in gene therapy. In particular, these molecules can modify the biological response during gene therapy in the skeletal muscle, resulting in an enhancement of the transgene expression as well as an enhancement of the therapeutic effect of the transgene. Furthermore, Pluronic® block copolymers are versatile molecules that can be used as structural elements of the polycation-based gene delivery systems (polyplexes). Based on these studies, the use of block copolymers in gene delivery is a promising area of research, in which new and important developments are expected.

Keywords: Gene delivery; Gene therapy; DNA; Polycation; Block copolymer; Poly(ethylene glycol)

Contents

1. Introduction

The field of non-viral gene therapy has recently gained increased interest [1]. It is widely believed that non-viral gene therapy can overcome some

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problems inherent to current viral-based therapies, including immune and toxic reactions as well as the potential for viral recombination [2]. One major approach in non-viral gene therapy is based on 'polyplexes', complexes formed by mixing DNA with synthetic polycations [3,4]. The polyplexes form spontaneously as a result of electrostatic interactions between the positively charged groups of the polycation and the negatively charged phosphate groups of the DNA. This results in DNA condensation, protection from the nuclease digestion, and more efficient delivery within a cell. A variety of polycation molecules have been proposed for polyplex formation [3,4]. These molecules differ in the chemical composition and the number of the repeating units, as well as in the architecture of the polymer backbone, which may be linear, randomly branched, dendrimeric, block- or graft copolymer. An alternative approach for gene delivery evaluates nonionic polymers, such as poly(vinyl pyrrolidone), which enhance gene expression of naked DNA in select tissues, such as skeletal muscle [5,6]. In contrast to polyplexes such nonionic polymers enhance gene expression through mechanisms, which most likely do not involve DNA condensation and facilitated transport within cells [7]. The current review describes a novel nonionic polymer class, Pluronic® block copolymers, which appear to be very valuable for gene delivery in skeletal muscle. Furthermore, Pluronic® block copolymers have proven to be useful elements in polyplexes on the base of the polycation and DNA complexes that have a potential in a variety of gene therapy applications. Both types of applications of the Pluronic® block copolymers for gene delivery are considered along with some major aspects of self-assembly and biological properties of these block copolymers, which are essential for the development of such gene delivery systems.

2. Structure and synthesis of Pluronic® block copolymers

Pluronic® block copolymers (also termed 'Poloxamer' or 'Synperonic') consist of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a triblock structure: EO_x–PO_y–EO_x. This arrangement results in an amphiphilic copolymer, in which the number of hydrophilic EO (x) and hydrophobic PO (y) units can be altered to vary the size, hydrophilicity and lipophilicity. The structure formula of Pluronic® block copolymers is presented in Fig. 1. Copolymers with various x and y values are characterized by distinct hydrophilic–lipophilic balance (HLB). The nomenclature of Pluronic block copolymers is explained in Appendix A.

Pluronic® block copolymers are synthesized by sequential polymerization of PO and EO monomers in the presence of an alkaline catalyst, such as sodium or potassium hydroxide [8]. The initial stage of the synthesis includes growth of the PO block followed by the growth of EO chains at both ends of the PO block. Anionic polymerization usually produces polymers with a low polydispersity index (M_n/M_w). However, the commercially available Pluronic® preparations may contain admixtures of the PO homopolymer as well as di- and triblock copolymers, exhibiting lower degrees of polymerization than expected. Chromatographic fractionation can be employed in procedures for the manufacture of highly purified block copolymers [9]. This reduces the presence of admixtures, particularly, of the PO homopolymer and of the block copolymers containing less of the EO block than expected.

3. Self-assembly of Pluronic® block copolymers

A defining property of Pluronic® is the ability of

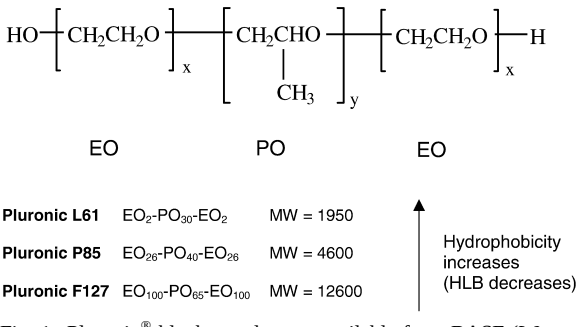

Fig. 1. Pluronic® block copolymers available from BASF (Wyandotte, MI), contain two hydrophilic EO blocks and a hydrophobic PO block.

individual block copolymer molecules, termed 'unimers', to self-assemble into micelles in aqueous solutions. These 'unimers' form a molecular dispersion in water at block copolymer concentrations below the critical micelle concentration (CMC). At concentrations of the block copolymer above the CMC, the unimer molecules aggregate, forming micelles through a process called '*micellization*'. The driving force for the micellization is the hydrophobic interactions of the PO blocks. The PO blocks self-assemble into the inner core of the micelles covered by the hydrophilic corona from EO blocks. Pluronic® micelles are commonly pictured as spheres composed of a PO core and an EO corona. This portrayal is correct for most block copolymers, which have an EO content above 30%, especially in relatively dilute solutions at body temperature. However, additional micelle morphologies, including lamella and rods (cylinders), can also form in Pluronic® systems [10]. When spherical micelles are formed, depending on the Pluronic® type the micelles commonly have an average hydrodynamic diameter ranging from about 20 to about 80 nm [10]. The number of block copolymer unimers forming one micelle is referred to as the '*aggregation number*'. Usually this number ranges from several to over a hundred.

The process of transfer of water-insoluble compounds into the PO core of the micellar solution is referred to as '*solubilization*'. Pluronic® micelles containing solubilized low molecular mass drugs and polypeptides are being actively investigated as potential drug delivery systems [11–19]. The core-shell architecture of polymeric micelles is essential for their utility for these applications. The core formed by the PO chains is a water-incompatible compartment that is segregated from the aqueous exterior by the hydrophilic chains of the EO corona, thereby forming, within the core, a 'cargo hold' for the incorporation of various therapeutic reagents. As a result, polymeric micelles can be used as efficient carriers for compounds, which alone exhibit poor solubility, undesired pharmacokinetics and low stability in a physiological environment. The hydrophilic shell contributes greatly to the pharmaceutical behavior of block copolymer formulations by maintaining the micelles in a dispersed state, as well as by decreasing undesirable drug interactions with cells and proteins through steric-stabilization effects.

4. Pluronic® block copolymers as biological adjuvants

In addition to the use of Pluronic® block copolymers as structural components of micellar drug formulations, Pluronic®-based systems exhibit a variety of useful biological properties. For example, water-in-oil, oil-in-water and water-in-oil-in-water emulsions formulated with select Pluronic® block copolymers have been used extensively as immunoadjuvants [20–31]. These studies suggested significant enhancement of both cell-mediated and humoral immune response induced by addition of the block copolymer formulations with respect to a very broad spectrum of antigens. Selected block copolymers, such as Pluronic® F127 have been found to significantly enhance the rate of wound and burn healing, and therefore have been included in cream formulations and skin substitutes for the treatment of burns and for other tissue engineering applications [32–39]. Several Pluronic®-based formulations were shown to effectively prevent postoperative adhesions or at least to reduce adhesion area after surgery [40,41]. Furthermore, Pluronic® block copolymers can enhance sealing of cell membranes permeabilized by ionizing radiation and electroporation thus preventing cellular necrosis, which can be helpful for improving drug and gene delivery in skeletal muscle [42–44]. Recent studies have demonstrated that select Pluronic® block copolymers can interact with multidrug resistant (MDR) cancer cells resulting in chemosensitization of these cells [45–49]. These interactions involve alteration of the structure of cell membranes by block copolymer molecules as well as energy depletion in MDR cells leading to the inhibition of various energy-dependent drug resistance mechanisms. Therefore, formulation of many anticancer drugs with such block copolymers results in enhancement of the chemotherapy of cancer [45,50]. These examples show that the block copolymers exhibit valuable biological activities, which can be of considerable importance for various therapeutic applications.

5. Enhancement of transgene expression in skeletal muscle by Pluronic®

One of the current and potentially successful

approaches in gene therapy involves the direct injection of Pluronic® block copolymers in combination with a plasmid DNA in skeletal muscle tissue, for example, to enhance immunization during DNA vaccination. It is well known that plasmid DNA can be injected into skeletal muscle and generate therapeutically meaningful levels of gene expression [51]. This application of naked DNA was demonstrated using therapeutic genes encoding (1) systemically acting secreted proteins, such as erythropoietin (EPO) and interleukin-5 [52,53], and (2) locally acting proteins, such as basic fibroblast growth factor, vascular endothelial growth factor and dystrophin [54–56]. However, in many cases the relatively low level of gene expression achieved limits the applicability of naked DNA as a therapeutic agent. Furthermore, commercially available cationic carriers, such as cationic dendrimers and lipids, that are known to improve gene expression in other tissues, significantly inhibit intramuscular gene expression (unpublished data).

Therefore, an alternative approach has been to identify compounds that can enhance gene expression in the muscle. One such compound, poly(vinyl pyrrolidone) has been widely discussed in the literature as a potential booster of the gene expression using the naked DNA [5–7,57–60]. This polymer does not condense the DNA at the concentrations used, which is a marked distinction compared to the cationic polymers, which all bind electrostatically to the DNA molecule and cause its condensation. Consequently, the mechanism of the poly(vinyl pyrrolidone) appears to be quite different from that of the polycations [7].

It was recently discovered that certain Pluronic® block copolymers significantly increase expression of plasmid DNA in skeletal muscle in mice [61]. A formulation based on the mixture of the block copolymers, Pluronic® L61 and Pluronic® F127, has been identified that increases gene expression by 5–20-fold compared to naked DNA. This formulation was termed SP1017.[1] Unlike cationic DNA carriers, and like poly(vinyl pyrrolidone), SP1017 does not condense DNA. Furthermore, this formulation does not improve the in vitro transfection of either the myoblast or the myofiber developmental stages of the murine muscle cell line, C2C12, when compared to plasmid DNA alone. However, for intramuscular administration, SP1017 was shown to promote expression of both reporter and therapeutic genes [61,62]. Importantly, the dose-dependency study suggested that maximal stimulation of gene expression with this formulation was observed at a relatively low concentration of the block copolymer (0.01% wt.), which provides for at least a 500-fold safety margin in animals. Recent data demonstrated that plasmid DNAs driven by either CMV- or NFκB-responsive elements were considerably more responsive to the SP1017 effect than plasmid DNAs that were under the control of either SV-40- or an AP-1-response element cassette [62]. Such promoter dependence is unusual and it suggests that SP1017 may act as a synthetic biological response modifier, probably by affecting mechanisms of the transcriptional control of the transgene expression. Compared to poly(vinyl pyrrolidone), SP1017 is more efficient and requires less DNA to produce the same amount of transgene [61].

The capacity of SP1017 to enhance plasmid-driven expression of secreted therapeutic genes was clearly illustrated in experiments carried out using the murine EPO gene [61]. The physiological effect that resulted from the expression of the transgene (mEPO) was readily quantifiable by simply measuring the hematocrit levels from the blood of the treated mice. In this study, the hematocrits of mice injected with 10 μg of the mEPO-expressing plasmid formulated with SP1017, increased to 58±4% compared to 45±2% observed in the control non-treated mice [61]. In contrast, the hematocrits of mice injected with the same dose of naked pCMV-mEPO (without SP1017 formulation) appeared slightly higher than those of the control non-treated animals, however the results were not statistically significant in this study.

Furthermore, the hematocrits of mice injected with pCMV-mEPO formulated with SP1017 were sustained for a long period of time, from 21 to 50 days post-injection. The increase in hematocrits obtained with pCMV-mEPO/SP1017-treated mice correlated with an increase in mEPO levels in both the serum and in the muscle, as detected by ELISA on day 7 post-injection. In contrast, animals treated with

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naked pCMV-mEPO showed a small, but significant increase in the mEPO level in muscle, while in the serum of these animals, the levels of mEPO remained below the sensitivity of the detection assay. The results of the dose escalation study showed that the hematocrits of mice treated with 50 μg of pCMV-mEPO/SP1017 reached 73±2%, which is comparable to the level achievable with adeno-associated virus [63].

While the mechanism of the effects of SP1017 in the applications described above is still being explored, it is noteworthy that the effects of SP1017 on transgene expression are observed in vivo only, using an animal model, but not in vitro, in cell culture. This suggests that SP1017 may exhibit its activity through interactions with some molecules or cells involved in the control of gene expression in the body, which are not present in vitro. Future studies should identify such targets of Pluronic® action. Furthermore, the fact that SP1017 exhibits maximal activity for intramuscular administration at a concentration that is close to the CMC values of both of the block copolymers present in the SP1017 mixture indicates that Pluronic® unimers are involved in this activity. In this respect, the molecular interactions underlying the activity of Pluronic® in gene therapy might be similar to the interactions involved in the block copolymer activity in MDR cells, such as effects on energy conservation mechanisms, changes in the structure and dynamics of biological membranes and effects on intracellular transport systems.

The toxicological aspects of injecting Pluronic® block copolymers in muscle tissue is of considerable interest in view of the gene delivery applications. The muscle toxicity, caused by injection of both single and multiple doses of various block copolymers, was evaluated by morphological examination of the muscle tissue and by monitoring creatine phosphokinase levels [64]. This study concluded that the toxicity of the block copolymers was proportional to their lipophilicity; the more lipophilic the copolymer, the more severe the lesions. Overall, Pluronic® F88 and Pluronic® F127 were considered suitable for incorporation into gel formulations, while Pluronic® P105 and Pluronic® P123 appeared to be more toxic than other accepted vehicles for intramuscular use, such as normal saline, Cremophor EL and peanut oil. It is noteworthy that the vehicles tested in this study contained 25% wt. Pluronic® gels, which is over a thousand times higher than the concentrations of block copolymers used in the gene delivery applications considered in this section. Therefore, as far as intramuscular administration of block copolymers is concerned, the Pluronic®-based gene delivery systems may have a very substantial safety margin. These studies demonstrate that Pluronic® block copolymers are promising agents for non-viral gene therapy. These agents provide for a simple and efficient gene transfer method potentially applicable in multiple gene therapy protocols involving intramuscular injection of plasmid DNA in order to synthesize therapeutic proteins or to vaccinate against a particular gene product.

6. Pluronic®-polycation conjugates for gene delivery

Some recent reports suggest that Pluronic® block copolymers can be useful as the components of novel self-assembling gene delivery systems. Astafieva et al. [65] have demonstrated that Pluronic® block copolymers can enhance polycation-mediated gene transfer in vitro. In this study, a synthetic polycation, poly(*N*-ethyl-4-vinylpyridinium bromide) was used to prepare complexes with a plasmid DNA, and then these complexes were evaluated for DNA intracellular uptake and transgene expression in cell culture models. Poly(*N*-ethyl-4-vinylpyridinium bromide) is a relatively low efficient gene transfer vector compared to some more recently developed polycations, such as polyethyleneimine (e.g. ExGen 500) or polyamidoamine dendrimers (e.g. Superfect™) [66]. However, when the poly(*N*-ethyl-4-vinylpyridinium bromide) and DNA were mixed with 1% Pluronic® P85 and then the cells were exposed to the resulting formulation, both the DNA uptake in the cells as well as the transgene expression were significantly increased compared to the cells treated with the poly(*N*-ethyl-4-vinylpyridinium bromide) and DNA complex alone [65]. A recent study reported that the receptor-mediated gene delivery to hepatic cell line, HepG2 using complexes of a plasmid DNA with an asialo-oroso-mucoid-poly(L-lysine) conjugate was increased fourfold in the presence of Pluronic® F127

[67]. Similarly, an additional increase of the transfection efficiency was demonstrated in the presence of Pluronic® F127 when the cervical cancer cell line, C-33A was transfected with the polycation/DNA complex [68]. It is interesting that Pluronic® F127 enhanced the transgene expression exhibited by the complexes whereas the agents that are frequently used to boost the DNA release from the endosomes, fusogenic peptide HA2 and chloroquine, had no effects on the transgene expression [67].

In another study, a block-graft copolymer synthesized by covalent conjugation of Pluronic® P123 and branched polyethyleneimine (P123-*g*-PEI(2K)) was evaluated as a gene transfer vector in vitro and in vivo [69]. This conjugate mixed with free Pluronic® P123 formed small and stable complexes with DNA (110 nm) that exhibited high transfection activity in vitro, comparable to that of Superfect™. Furthermore, gene expression was observed in spleen, heart, lungs and liver 24 h after intravenous injection of this complex in mice. Compared to 1,2-bis(oleoyloxy)-(trimethylammonio) propane (DOTAP)-cholesterol and branched polyethyleneimine (25 kDa) transfection systems, the block-graft copolymer system revealed a more uniform distribution of gene expression between these organs, allowing for a significant improvement of gene expression in liver.

A recent study evaluated several non-viral transfection systems based on the complexes of DNA and polycations (polyplexes) with respect to their effectiveness, toxicity and cell type dependence in a variety of in vitro models [66]. The panel of polycations examined included branched and linear polyethyleneimines (25 kDa and 50 kDa), poly(*N*-ethyl-4-vinylpyridinium bromide), polyamidoamine dendrimer (Superfect™), poly(propyleneimine) dendrimer (Astramol™) and a P123-*g*-PEI(2K) graft-block copolymer. Using a panel of cell lines the linear polyethyleneimine ExGen™ 500, Superfect™, branched polyethyleneimine 25 kDa, and P123-*g*-PEI(2K) were determined as systems displaying highest transfection activity while exhibiting relatively low cytotoxicity. These systems had activity higher than or comparable to lipid transfection reagents (Lipofectin®, LipofectAMINE™, CeLLFECTIN® and DMRIE-C) but did not reveal serum dependence and were less toxic than the lipids. This study demonstrated good potential of P123-*g*-PEI(2K) graft-block copolymer based polyplex system as transfection reagents with relatively low cytotoxicity. As is shown in Fig. 2 this system

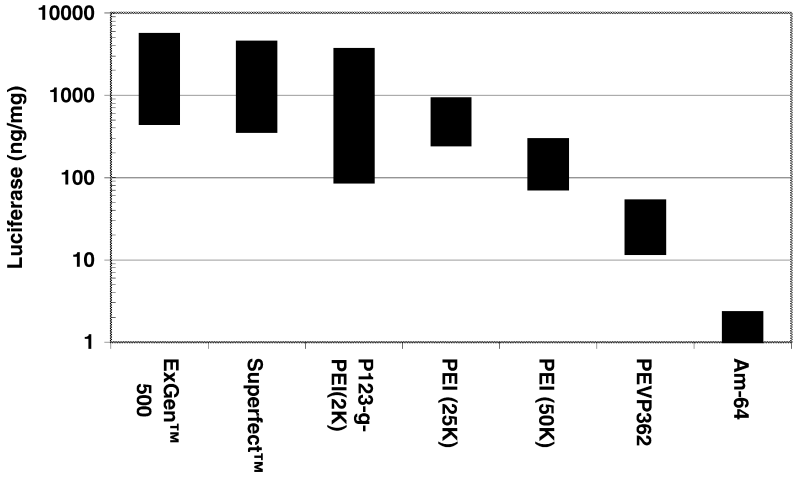

Fig. 2. Relative transfection activity of various polyplexes in Cos-7 cells using luciferase reporter gene. Data represents the range from the lowest to the highest values of luciferase expression (ng/mg) obtained following transfection with a given polyplex over a series of experiments [66]. PEI, polyethyleneimines; PEVP, poly(*N*-ethyl-4-vinylpyridinium bromide), polyamidoamine dendrimer (Superfect™); Am-64, Astramol™.

ranks among the best in vitro transfection systems. One major advantage of this system is that the complexes formed by P123-*g*-PEI(2K) and DNA in the presence of the free Pluronic® are stable in a variety of conditions particularly in the presence of serum proteins [69]. In contrast, the complexes formed by homopolymer polyethyleneimine or polyamidoamine dendrimers reveal the tendency for aggregation particularly in the presence of serum (Table 1).

High stability of the block-graft copolymer-based complexes is evidently due to formation of the micelle-like structures, which include the DNA, polycation and block copolymer chains. Electrostatic binding of the DNA and polyethyleneimine chains of P123-*g*-PEI(2K) results in neutralization of the polyion charges. The zeta-potential of resulting complexes is close to zero, yet the complexes form stable dispersions. Therefore, it is likely that these systems are stabilized in dispersion by the EO corona in a manner similar to regular Pluronic® micelles. Furthermore, the self-assembly of these materials is likely to involve the hydrophobic interactions of the PO chain segments. It is noteworthy, that the free Pluronic®, a component shown to be essential for exhibiting high transfection activity, is also necessary for formation of a stable dispersion from DNA and P123-*g*-PEI(2K) [70].

7. Pluronic®-polycation conjugates for delivery of oligonucleotides

Beside plasmid DNA, block copolymers have been used to formulate and deliver oligonucleotides [71–76]. In particular, cationic copolymers, such as, for example, poly(ethylene oxide)-*g*-polyethyleneimine, PEO(8K)-*g*-PEI(2K), spontaneously form polyelectrolyte complexes with phosphorothioate and phosphodiester oligonucleotides. These structures are described as core–shell micelle-like particles with a core from the neutralized oligonucleotide/polycation complex and a shell from poly(ethylene oxide) chains attached to polyethyleneimine. This type of system has been successfully used for the in vivo delivery of oligonucleotides [74]. More recently, a block-graft copolymer of Pluronic® P85 and polyethyleneimine, P85-*g*-PEI(2K), was used for preparing oligonucleotide formulations [77]. Like in the case of the plasmid DNA described in the previous section the oligonucleotide formulation also contained free Pluronic® P85 to stabilize the dispersion. The organ accumulation of antisense oligonucleotides formulated with P85-*g*-PEI(2K) was recently examined. Following i.v. administration oligonucleotide formulated with PEO(8K)-*g*-PEI(2K) accumulated mainly in kidneys, while the same oligonucleotide formulated with P85-*g*-PEI(2K) was found almost exclusively in the liver. Furthermore, in the case of the animals injected with the P85-*g*-PEI(2K)-based complexes most of the ODN was found in hepatocytes, while only a minor portion of oligonucleotide was found in the lymphocyte/monocyte populations. The results of this study suggest that formulating oligonucleotide with PEO(8K)-*g*-PEI(2K) and P85-*g*-PEI(2K) carriers allows to redirect ODN to the liver or kidneys, respectively. The variation in the tissue distribution of ODN observed with the two carriers is probably due to different hydrophilic–lipophilic balances of

Table 1
Effect of buffer components and serum on the size of the polyplexes [69]

Polycation	Buffer	Particle diameter, nm	
		+ Serum	− Serum
Polyethyleneimine, 25 kDa	10 mM phosphate	850	1100
	PBS	460	380
	DMEM	580	140
P123-*g*-PEI(2K)	10 mM phosphate	260	250
	PBS	270	270
	DMEM	320	215

the polyether chains grafted to polyethyleneimine in these molecules. Therefore, cationic block copolymers provide a simple way to enhance oligonucleotide accumulation to selected compartments without the need of a specific targeting moiety.

Thus these systems appear to be similar to polyion complex micelles from DNA and cationic block copolymers proposed for gene delivery by several investigators, which have a core from neutralized DNA and polycation chains and a corona from hydrophilic nonionic chains such as EO. However, in the case of P123-*g*-PEI(2K)-based complexes, the nonionic Pluronic® chains also carry hydrophobic PO components. The PO chains in Pluronic® molecules are known to interact with lipid membranes and induce structural rearrangements in the membranes. Furthermore, due to the presence of PO chains Pluronic® molecules exhibit the ability to translocate within cells, while homopolymer EO does not exhibit this ability. Thus, P123-*g*-PEI(2K)-based complexes contain 'sticky' elements, which, in essence, are similar to fusogenic sequences in the viruses, that allow for effective delivery of genetic material within a cell. Due to the presence of such elements P123-*g*-PEI(2K) copolymer is much more efficient in transporting DNA within the cells compared to the block- and graft-copolymers formed by polycation and EO chains [70]. Furthermore, the polycations modified by EO chains, e.g. poly(ethylene oxide)-*g*-polyethyleneimine, are usually much less efficient transfection agents than the unmodified polycations, e.g. polyethyleneimine. In contrast, the polyplexes formed by P123-*g*-PEI(2K) copolymer have practically the same or comparable activity as the unmodified polycation. Overall Pluronic®-based polyplexes, such as the P123-*g*-PEI(2K) graft-block copolymer complexes, are promising vectors for gene delivery, which have significant advantages compared to many other polyplex systems. These advantages result from the fact that the Pluronic®-based polyplexes combine (1) high stability in dispersion with (2) high transfection activity.

8. Conclusion

In conclusion, although the studies described in this paper are relatively recent, the results obtained already suggest that Pluronic® block copolymers are promising agents for utilization in gene therapy applications. Firstly, it appears that a least some of these molecules can modify the biological response during gene therapy, resulting in an enhancement of the transgene expression as well as an enhancement of the therapeutic effect of the transgene. Secondly, Pluronic® block copolymers are versatile molecules that can be used as structural elements in novel self-assembling gene delivery systems that may be superior compared to currently known vectors. Based on these studies, the use of block copolymers in gene delivery is a very exciting area of research in which new and important developments are expected in the near future.

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Appendix A. Nomenclature of Pluronic® block copolymers

Pluronic® nomenclature includes one letter, 'F', 'P', or 'L', followed by a two- or three-digit numeric code. The letters stand for solid ('F'), paste ('P') or liquid ('L'). The numeric code defines the structural parameters of the block copolymer. The last digit of this code approximates the weight content of EO block in tens of weight percent (for example, 80% wt. if the digit is 8 or 10% wt. if the digit is 1). The remaining first one or two digits encode the molecular mass of the central PO block. To decipher the code, one should multiply the corresponding number by 300 to obtain the approximate molecular mass in Da. Therefore Pluronic® nomenclature provides a convenient approach to estimate the characteristics of the block copolymer in the absence of reference literature. For example, the code 'F127' defines the block copolymer, which is a solid, has a PO block of 3600 Da (12×300) and 70% wt. of EO. The precise molecular characteristics of each Pluronic® block

copolymer are provided by the manufacturer and can also be located in the literature [19].

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