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Protein release from polyphosphazene matrices

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Abstract

Polyphosphazenes have been exploited as carriers for protein delivery due to versatility of molecular structures and sophisticated spectrum of chemical and physical properties. Ease of structural manipulations for this class of organometallic polymers allows efficient control over physico-chemical parameters of polyphosphazenes including their biodegradability and matrix permeability. Some polyphosphazenes offer additional advantages as protein delivery vehicles since microencapsulation of substrates in these systems can be achieved under remarkably mild physiological conditions. Because of these properties polyphosphazenes have tremendous potential as matrices for protein release as shown by studies both in vitro and in vivo.

Keywords: Polyphosphazenes; Controlled release; Proteins; Biodegradation; Hydrogels

Contents

1. Introduction

In recent years one of the most versatile types of synthetic biomaterials has been a relatively new class of organometallic polymers — polyphosphazenes. Polyphosphazenes are high molecular weight polymers containing a long-chain backbone of alternating phosphorus and nitrogen atoms with two organic side groups attached to each phosphorus atom (**1**). Fundamental developments in polyphosphazene synthesis yielded synthetic pathways allowing a choice of a tremendous variety of substituents. Because of this

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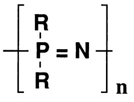
synthetic flexibility phosphazene polymers exhibit a very broad and sophisticated spectrum of chemical and physical properties that make them suitable for many applications. Biomedical applications for these polymers can range from materials for cardiovascular and dental uses to implantable and controlled release devices [1,2]. This chapter will be devoted to a review of polyphosphazenes as carriers for protein delivery systems.



$$\left[-\overset{\displaystyle R}{\underset{\displaystyle R}{P}}=N- \right]_n$$

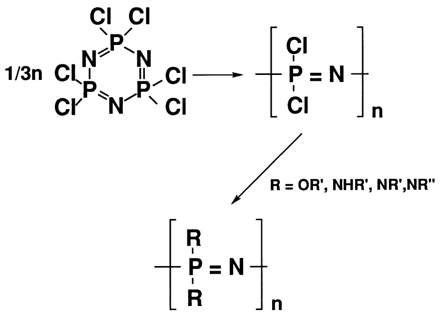
(1)

2. Polyphosphazenes as materials for controlled release technologies

2.1. Advances in polyphosphazene synthesis

Biomedical applications usually require control of macromolecular structure and molecular weight distribution and thus it is important to discuss briefly the current state of polyphosphazene synthesis and also recent advances made in this field. Polyphosphazene synthesis is generally more involved than that of common petrochemical polymers, however, it also offers a unique synthetic flexibility not available for most other classes of polymers.

The best studied and most important route to polyphosphazenes is a macromolecular substitution route [3], in which a reactive polymer, poly(dichlorophosphazene) is used as an intermediate in the synthesis of stable substituted polyphosphazenes (Scheme 1). Poly(dichlorophosphazene) is usually synthesized by a thermal ring opening cationic polymerization of hexachlorocyclotriphosphazene in bulk at 250°C and has high molecular weight (10^6 g/mol) and relatively broad molecular weight distribution. Lewis acids can be used as catalysts and this process usually yields polymer of a lower molecular weight and allows limited control over the molecular weight.



Scheme 1.

An alternative method of poly(dichlorophosphazene) synthesis is a solution polycondensation of *P*-trichloro-*N*-(dichlorophosphoryl)monophosphazene, $Cl_3P{=}NP(O)Cl_2$ at 200°C which also produces polymers with some control over molecular weights [4]. A new access route to poly(dichlorophosphazene) was discovered recently which allows the synthesis of this macromolecular reactant by 'living' cationic polymerization of phosphoranimines at ambient temperature and can potentially provide polymers with controlled molecular weights, narrow polydispersities and also be used for the preparation of block copolymers [5].

Stable substituted polyphosphazenes are synthesized by the reaction of poly(dichlorophosphazene) with organic nucleophiles, such as sodium salts of alcohols or phenols, or with primary or secondary amines to replace chlorine atoms with organic groups. Significant advances were made in the study of these reactions, especially in the synthesis of copolymers where the effect of reaction conditions and the structure of nucleophile on the polymer composition and side group arrangements were investigated [6]. Methods of direct synthesis of poly(aryl/alkyl)phosphazenes via condensation polymerization of *N*-silylphosphoranimines [7] and polyphosphazenes with trifluoroethoxy groups using anionic polymerization of phosphoranimines [8] were also described.

2.2. Modulating physico-chemical properties of polyphosphazenes for controlled release applications

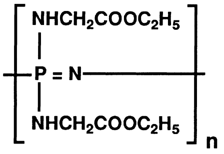
The macromolecular substitution route allows preparation of polyphosphazenes of almost unlimited structural varieties including mixed substituent copolymers in which up to several different side groups can be introduced in a polymer structure by simultaneous or sequential substitution. Since physical and chemical properties of polyphosphazenes are determined to a great extent by polymer side groups, polyphosphazenes display a very diverse set of physico-chemical characteristics, which can be tailored with a remarkable ease. However, the uniqueness of polyphosphazenes is not only in synthetic versatility but also in the presence of phosphorus-nitrogen backbone, which can be rendered hydrolytically unstable when combined with appropriate side groups, leading to almost unlimited possibilities in preparation of biodegradable materials [1], or serve as an ideal starting point for designing hydrogel matrices with unusual hydrophilic backbone [9].

2.2.1. Biodegradable polyphosphazenes

The ability of hydrophobic polymer matrices to degrade slowly in aqueous solutions is one of the most critical properties in determining the potential of these materials as controlled release systems. Degradable solid hydrophobic polymers are now being used in animal studies to release proteins, polysaccharides and polynucleotides [10]. Since Allcock reported that amino-substituted polyphosphazenes are susceptible to hydrolytic degradation and hold promise as biodegradable matrices [11,12], a search is under way for polyorganophosphazenes with controlled rate of degradation and good biocompatibility. To date, a number of hydrophobic biodegradable polyphosphazenes were evaluated as potential candidates for drug delivery systems including polyphosphazenes containing imidazolyl [13], ethylamino [14], oligopeptides [15], amino acid esters, such as ethyl alanato, benzyl alanato [16], ethyl glycinato [16,17] and depsipeptide groups (dimers composed of an amino acid and a glycolic or lactic ester) [18] as hydrolysis sensitive side groups.

Exhaustive investigation of solid state hydrolysis of poly[(amino acid ester)phosphazenes] in aqueous solution was performed recently using methyl, ethyl, tert-butyl, and benzyl ester derivatives of glycine, alanine, valine, and phenylalanine as side groups of poly(organophosphazenes) [19]. It was shown that the larger the group linked to the α-carbon atom of the amino acid residue and the bulkier the ester end group, the less hydrolytically sensitive was the polymer. In general, phenylalaninato units were more stable than the valinato units which were more stable than the alaninato groups, and with the glycinato side groups being the least stable to hydrolysis. The benzyl esters were less sensitive than the tert-butyl esters, which were less sensitive than ethyl esters, and the methyl esters were the most hydrolytically unstable.

Study of hydrolytic decomposition of three polyphosphazenes sharing the same amino acid residue or the same ester group was conducted in order to investigate the possibility of tailoring the release profiles by changes in the structure of the polymer side groups [16]. Poly[di(ethyl glycinato)phosphazene] (**2**), poly[di(ethyl alanato)phosphazene] (**3**) and poly[di(benzyl alanato)phosphazene] (**4**) were incubated in aqueous borate buffer (pH 7) and the mass loss, as well as molecular weight decline were measured over time. Poly[di(ethyl glycinato)phosphazene] (**2**) showed the fastest decomposition, followed by polymer (**3**) and then polymer (**4**). As in the previously described study these results indicate that the rate of hydrolysis is mainly determined by the substituent at the α-carbon (a methyl group or a hydrogen atom) of the amino acid ester side group. The presence of a methyl group in this position in polymers (**3**) and (**4**) provides better steric protection against hydrolytic attack on the phosphazene backbone or on the side group phosphorus nitrogen bond explaining lower hydrolytic sensitivity of these polymers compared to poly[di(ethyl glycinato)phosphazene] (**2**). The type of ester group (ethyl or benzyl) had apparently less bearing on the rate of hydrolysis, but was still important with respect to the surface properties of polymer matrices. It appeared that surface hydrophobicity of polymer matrices, as examined by contact angle measurements, was significantly higher for polymer (**4**), and in contrast to polymers (**2**) and (**3**) practically did not change during the degradation experiments. Thus, the presence of a bulky and hydrophobic ester group can significantly reduce water penetration in a polymer matrix and increase hydrolytic stability of the polymer. Release profiles of small molecules from these polymers were also analyzed and it was concluded that polymers that degrade faster also release guest molecules faster. Two modes of release were observed — the initial release appeared to be through diffusion of the drug through the polymer matrix and the release of the drug during the second phase can be viewed as a combination of diffusion and erosion, but dominated by release through polymer erosion.



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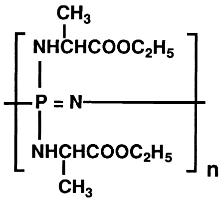

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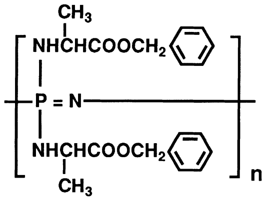
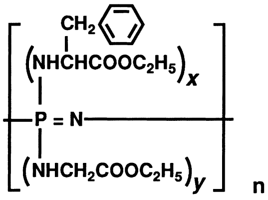


(4)

Direct correlation between the ability of mixed substituent polyphosphazenes to degrade and release encapsulated material and their composition was demonstrated recently using mixed substituent polyphosphazenes containing ethyl phenylalanato and ethyl glycinato side groups (**5**) [20]. The results of release experiments performed in PBS at 37°C using the cytostatic agent mitomycin C as an encapsulated drug showed significant increase in the release rates with the increase in the amount of less hydrophobic and more hydrolytically sensitive ethyl glycinato groups. Release experiments conducted for matrices prepared by blending polyphosphazenes of different compositions demonstrated a similar increase in the release rates for matrices containing polymers with higher content of ethyl glycinato groups.



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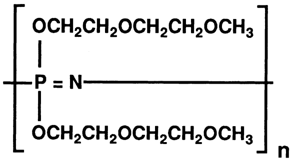
2.2.2. Polyphosphazene hydrogels

Polymer hydrogels represent another class of synthetic materials that found extensive applications for controlled release of drugs [21,22]. These materials are capable of retaining a significant amount of water within their structure, which makes them permeable to water-soluble molecules, including proteins [10,21]. The release is controlled by the macromolecular structure of the carrier as defined by polymer nature, degree of cross-linking and related parameters [21]. The attractiveness of polyphosphazenes in designing water-soluble polymers and hydrogels is that the polymer hydrophilicity is derived not only from the presence of a hydrophilic side group, but also results from the hydrophilicity of polyphosphazene backbones that possess lone pair electrons for hydrogel bonding to water. The hydrophilic nature of the phosphazene backbone in combination with the already mentioned ease of structural manipulation for polyphosphazenes leads to interesting physico-chemical properties [9].

Polyphosphazene hydrogels, as any other synthetic hydrogels can be divided into two major classes — neutral or non-ionic hydrogels and ionic hydrogels, those formed by phosphazene polyelectrolytes — macromolecules bearing pendant ionic or ionizable groups. Non-ionic polyphosphazene hydrogels can be based on water-soluble polymers ranging from ultimately hydrophilic, such as polyphosphazene containing glucosyl and glyceryl side groups, to less hydrophilic including poly[bis(methylamino)phosphazene], for which solubility is believed to be partially due to the exposure of skeletal nitrogen bonding to water [9].

One of the most interesting examples of polyphosphazenes with amphiphilic side groups are poly[bis(methoxyethoxyethoxy)phosphazene] (MEEP) (**6**), related polymers and their hydrogels [23,24]. MEEP, and a number of polymers related to MEEP are only water-soluble below a specific temperature — low critical solution temperature (LCST), but precipitate from solution as the temperature is raised above LCST. Since LCST varies depending on the structure of side groups, polymer composition and is also sensitive to the ionic strength [25], MEEP-based polymers and hydrogels can be fine tuned for specific temperature and ionic strength responsive drug release applications. Another advantage of these types of polymer is that their hydrogels can be easily prepared by exposure to gamma rays or ultraviolet light [24,26], and the physical properties can be modified by variations in radiation doses. Diffusion of small molecules in these matrices was investigated showing the potential of MEEP hydrogels for controlled release applications [24].

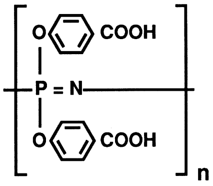


$$\left[\begin{array}{l} OCH_2CH_2OCH_2CH_2OCH_3 \\ | \\ -P=N- \\ | \\ OCH_2CH_2OCH_2CH_2OCH_3 \end{array} \right]_n$$

(**6**)

Ionic polyphosphazene hydrogels — hydrogels formed by polyphosphazene polyelectrolytes have been extensively studied recently as matrices for controlled delivery devices. In contrast to most traditional synthetic polyelectrolytes, polyphosphazene polyelectrolytes are usually bearing two charged groups per repeating polymeric unit instead of one, and thus relatively high charge density can be achieved for these molecules. Since higher density of ionised groups favors, in general, water-solubility or hydrogel swelling properties of polyelectrolytes, a greater extent of hydrophobicity can be potentially built in these molecules without rendering the polymer water-insoluble. Thus, important characteristics of ionic hydrogels, such as an ability to respond to the environmental changes, such as pH and ionic strength can be modulated even in a broader range for polyphosphazene polyelectrolytic gels.

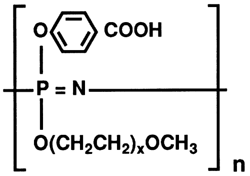
Perhaps, the most studied polyphosphazene polyelectrolyte-poly[di(carboxylatophenoxy)phosphazene], or PCPP (**7**) has two hydrophobic phenoxy side groups per repeating unit with two carboxyl groups in *para*-position, that render it water-soluble when ionized in neutral and basic solutions. It was originally synthesized as a potential material for the preparation of membranes [27], but was immediately selected as an ideal candidate for microencapsulation of proteins and mammalian cells [28–30] because of its ability to form soft, highly swollen hydrogels in the presence of salt of divalent cations under mild physiological conditions. Later it was shown that it is also a powerful immunostimulant, and is particularly effective when used as a vaccine delivery vehicle [31–33].



(**7**)

PCPP behavior in aqueous solutions was investigated, and its hydrophobic properties and high sensitivity to pH and ionic composition of solution were reported [27,30,34–38]. It was also demonstrated that gel-forming properties of PCPP, structure and permeability of ionotropic hydrogels based on PCPP can be effectively controlled by the type and concentration of ionic cross-linker, pH and ionic strength of solution, and by preparation of a less permeable and more rigid surface coating formed by polyelectrolyte complexes between PCPP and polycations, such as poly(L-lysine) [28–30].

Another approach to a modification of physicochemical properties of carboxyl-acid-containing hydrogels — synthetic derivatization of PCPP was also developed. A number of mixed substituted polyphosphazenes (**8**) having an improved solubility in aqueous solutions was synthesized and characterized [36,39]. The possibility of synthesis of carboxyl-acid-containing hydrogels with stable covalent cross-linkers was also demonstrated. Poly[(carboxylatophenoxy)(methoxyethoxyethoxy)phosphazenes] were cross-linked by ^{60}Co gamma irradiation with an objective to prepare hydrogels swellable in a broad pH range and sensitive to pH and ionic strength changes [39]. These hydrogels were also permeable to small molecules both at low and neutral pH with much higher diffusion rates at neutral pH. Hydrolytic instability is another property that can be introduced in ionic polymer hydrogels by synthetic modification of polyphosphazene. Arylcarboxylic acid derivatives containing glycinato side groups, as hydrolytically sensitive co-substituents, were synthesized [40]. Microspheres formed by these polymers showed greater potential to degrade under physiological conditions.



(**8**)

An interesting approach to the synthesis of biodegradable phosphazene-containing hydrogels has been reported recently [41]. The method makes use of a degradable trimeric phosphazene derivative as an effective network-forming molecule. A phosphazene cross-linker having substituted allylamino groups and imidazole groups was copolymerized with acrylic acid to form a hydrogel degradable in an aqueous solution over a period of 1–2 months. By varying the ratios of the two phosphazene substituents, it should be possible to vary the degradation rate. According to the authors this synthetic concept can be utilized with any acrylates or other vinyl monomers and even be extended to preparing hydrolytically unstable biopolymer networks.

2.3. Microencapsulation in polyphosphazene matrices

Adaptation of microencapsulation methods to sensitive biomolecules, such as proteins, peptides and antigens is one of the challenges in the development of protein delivery systems, since special care should be taken to avoid denaturation or loss of biological activity of encapsulated material [42]. Many traditional microencapsulation techniques, such as spray-drying or solvent evaporation can be applied to preparation of microspheres with hydrophobic polyphosphazene matrices [43], however it is usually difficult, if not impossible to avoid elevated temperatures or at least partial exposure of protein to organic solvents used in the process.

The newer developments in polyphosphazene microsphere preparation include methods which utilize the already mentioned ability of certain polyphosphazene polyelectrolytes, such as PCPP (**7**) to form ionotropic hydrogels in aqueous environment under mild physiological conditions in a manner similar to a gelation of some natural polymers, such as alginate. The key element of this technique is a generation of microdroplets of polymer- and protein-containing aqueous solution, which are then ionically cross-linked with salts of divalent cations to form stable polyphosphazene microspheres. Originally, microdroplets were prepared using an apparatus consisting of a syringe pump, plastic syringe and air-jet syringe nozzle which generated microspheres in the size range between 0.5–1.5 mm [28,30]. Later the process was improved to produce microspheres with diameters in the 1–10 μm range by replacing the syringe nozzle with an ultrasonic spray nozzle [32].

Recently it was observed that PCPP solutions can form coacervate systems, consisting of fine droplets of polymer solution and a polymer deficient phase, when treated with solutions of sodium chloride [38]. Microdroplets effectively retain protein dissolved in polymer solution and can be then permanently stabilized to form microspheres with addition of calcium chloride. Essentially it was demonstrated that the addition of sodium chloride to a polymer solution is similar to the role of a nozzle and thus the use of the spraying technique can be completely avoided. The advantages of the aqueous coacervation method are that it not only eliminates the need for complicated equipment and generation of aerosols, but also that it enables much more effective control over microsphere sizes in the 1–100 μm range by simply varying the concentration of the components. Coacervation microspheres also have more narrow particle size distribution compared to the previous techniques, and contrary to spray microspheres do not contain noticeable amounts of larger size aggregates or amorphous precipitates. The technology was successfully applied to the encapsulation of proteins, such as bovine serum albumin and influenza antigen with an encapsulation efficiency of more than 90% [38].

3. Protein release from polyphosphazene matrices

3.1. Protein release from biodegradable hydrophobic polyphosphazenes

Hydrolytically unstable hydrophobic polyphosphazenes offer distinct advantages for use as bioerodible controlled drug delivery devices. One of these advantages is the already mentioned ease of control over hydrolytic instability of polymer matrices in these systems.

The feasibility of biodegradable polyphosphazenes for use as matrices for protein release was demonstrated using polyphosphazenes with imidazolyl and methylphenoxy side groups [13]. Mixed substituent polyphosphazene copolymers containing methylphenoxy and hydrolytically unstable imidazolyl groups were evaluated as matrices for release of *p*-nitroaniline, progesterone and ^{14}C-labeled bovine serum albumin (BSA) both in vitro and in vivo. It was found that the degradation profiles of polymeric devices were controlled by the relative amount of imidazolyl groups present in the polymer. The rate of degradation of poly[(imidazolyl)(methylphenoxy)phosphazene] containing 45% of imidazolyl groups in an aqueous medium was significantly higher than that of copolymer containing only 20% of hydrolytically unstable imidazolyl groups. Release characteristics of the hydrophobic bioerodible polymeric devices were determined, however, not only by their degradation profiles, but also by the type of the encapsulated material. Contrary to the release of low molecular weight substrates, the release profile for BSA loaded in the polymer containing the same amount of imidazolyl groups had a 'burst release' stage when almost 25% of protein diffused out of the matrix suggesting possibility of polymer matrix modification in the presence of protein. A successful attempt was made to minimize the 'burst release' stage by coating BSA loaded matrices with 20% polyphosphazene solution in THF. Coated matrices released only 5% of protein during the first 4 h.

Similar changes in properties of polyphosphazene matrices caused by encapsulation of macromolecular substrates were observed for another biodegradable hydrophobic polyphosphazene — poly[(*p*-methylphenoxy)(ethyl glycinato)phosphazene] [44]. This mixed substituent copolymer containing 50% of *p*-methylphenoxy and 50% of hydrolytically unstable ethyl glycinato groups was investigated as a matrix for controlled release of macromolecules using inulin, a polysaccharide with a molecular weight of 5000 g/mol. As can be expected, a correlation was observed between inulin release and degradation of polyphosphazene. Inulin release was highest at pH 2.0 and lowest at pH 7.4 following the trend for polyphosphazene degradation. As in the previous example, polymer degradation and controlled release of the macromolecular substrate were greatly affected by the relative amount of material loaded in the polymer matrix. In general, increasing the loading apparently results in increased matrix hydrophilicity translating to higher degradation and more rapid release, with highest release and pronounced 'burst effect' for polymer loaded with 40% of inulin. These results correlate with changes in surface appearances of these samples observed using environmental scanning electron microscopy. The surfaces of polyphosphazene matrices without inulin appeared fairly smooth while polymers loaded with the macromolecular substrate had rough, dense and granular surfaces.

One of the other important advantages of biodegradable hydrophobic polyphosphazenes for use as controlled release matrices is also that these materials show good biocompatibility. Poly[(imidazolyl)(methylphenoxy)phosphazene] matrices implanted subcutaneously in rats were found to show no gross areas of surrounding inflammation at the time of explantation [13].

3.2. Protein release from polyphosphazene hydrogels

Polyphosphazene hydrogels were extensively tested as drug delivery vehicles since methods of their preparation, particularly ionotropic gelation of PCPP, allow extremely mild conditions of microencapsulation. Diffusion remains the most important mechanism for protein release from hydrogels [22] and, thus, permeability of the PCPP hydrogels was evaluated using proteins with varying molecular weights and model substrates — fluorescent polystyrene microbeads [30]. The release kinetics from PCPP microspheres was studied as a function of conditions of microencapsulation and formation of polyelectrolyte membranes on the surface of the gel microspheres. It was demonstrated that the polymer concentration used in the preparation of microspheres can significantly affect the outward diffusion of the macromolecular substrates. For example, increasing the PCPP concentration from 1.5% to 3.3% (w/w) led to a more than fourfold decrease in BSA release after 24 h [30]. An increase in calcium ion concentrations also tended to decrease substrate release rates.

Another way to control the permeability and substrate diffusion rates from polyphosphazene microspheres is the creation of a semipermeable membrane by coating microspheres with a polyelectrolyte complex formed by PCPP and poly(L-lysine) [30]. The comparative release studies for PCPP with and without coating was performed in PBS (pH 7.4) at 37°C using FITC-BSA as substrate. The results showed that poly(L-lysine) coating decreased microsphere permeability and sustained protein release. Uncoated microspheres released more than 40% of the FITC-BSA during the first half hour compared to

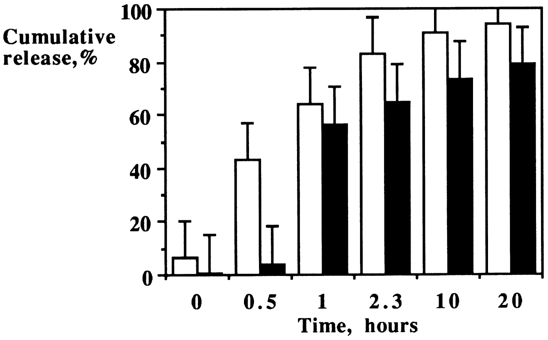


Fig. 1. Cumulative FITC-BSA release from PCPP microspheres (□) and the same microspheres coated with PLL of M_w 21.5 kDa (■). Microspheres were prepared from 2.5% (w/w) polymer solution and then coated with 0.025% (w/w) PLL solution during 30 min. Protein release was measured in PBS (pH 7.4) at 37°C. (From [30]).

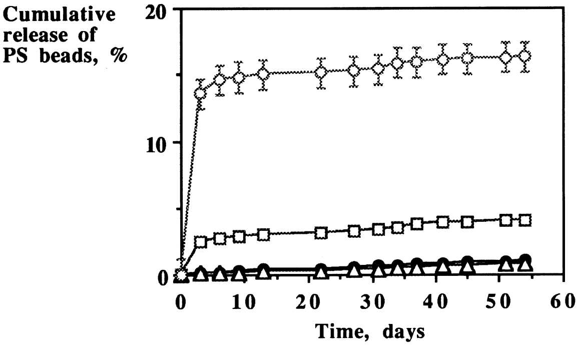


Fig. 3. Release of PS beads from PCPP microspheres coated with PLL of different molecular weight: (●) 12 kDa, (△) 62.5 kDa, (□) 140.8 kDa, (○) 295 kDa. Concentration of PLL solution was 0.04% (w/w), and the reaction time 6 min. The coated microspheres were then incubated in PBS as in Fig. 2. (From [30]).

~5% of the protein released from microspheres coated with low molecular weight poly(L-lysine) (Fig. 1). The permselectivity of PCPP-poly(L-lysine) membranes for macromolecules of different molecular weights was also demonstrated. While almost 70% of the FITC-BSA diffused out during the first 5 h, only 20% of β-gal was released over the same time period (Fig. 2).

The effects of poly(L-lysine) molecular weight, concentration and time of polyelectrolyte complex formation on the permeability of poly(L-lysine) coated microspheres were investigated. An increase in the molecular weight of polycation above 60 kDa not only significantly increased the release rate of model polystyrene microbeads, but also changed the release profile to a bi-phasic pattern by adding an initial 'burst' from microspheres coated with high molecular weight poly(L-lysine) (Fig. 3). A similar 'burst' effect was found when the concentration of poly(L-lysine) in the coating step was increased. These results were explained by the presence of two different types of cross-linker in the polymer matrix: (a) 'ionic bridges' formed by calcium ions, which are extremely sensitive to the ionic environment due to the ion exchange reactions between divalent and monovalent ions, and (b) cross-links caused by formation of polyelectrolyte complexes (PCPP-poly(L-lysine)) which are relatively stable under the conditions studied [45,46]. The cross-linker distribution is determined by its diffusion in the matrix during gel formation, which is severely limited for high molecular weight poly(L-lysine). The latter can apparently only form a thin superficial layer of a stable complex, with the remaining part of the microsphere consisting of highly ionically sensitive matrix, leading to a higher degree of swelling observed for these microspheres. It was hypothesized that high osmotic pressure in these systems can lead to a rupture of the relatively rigid PCPP-poly(L-lysine) membrane 'shell' in the same way as is observed for solvent-activated drug delivery capsules [10] causing a 'burst effect' on the release profiles. Following membrane rupture, and after termination of polymer swelling, redistribution of poly(L-lysine) on the surface of microspheres can occur with re-establishment of a uniform coating that allows only a

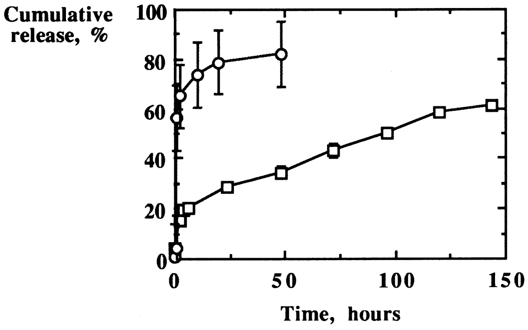


Fig. 2. Cumulative release of FITC-BSA (○) and β-galactosidase (□) from microspheres coated with 0.025% PLL solution (21.5 kDa) during 30 min. Protein release was measured in PBS (pH 7.4) at 37°C. (From [30]).

slow diffusion of substrate through the PCPP-poly(L-lysine) membrane.

Thus it was demonstrated that hydrogel permeability and release patterns can be effectively modulated by selecting different formulation parameters, such as polyphosphazene and ionic cross-linker concentration, or to a greater extent, by varying the conditions of their coating with the polycation.

4. PCPP as a vaccine delivery vehicle

One application of the controlled release of proteins that has sparked considerable interest recently is the development of vaccines that would require a single administration since the major disadvantage of several currently available vaccines is the need for repeated administrations. Microencapsulation has been applied to the administration of vaccines both by the parenteral and mucosal routes. A frequent choice of a carrier for these vaccines is poly(D,L-lactide-co-glycolide) (PLGA), a biodegradable polyester that has a long history of medical use in erodible sutures, bone plates and other temporary prostheses. In the last few years, a body of data has accumulated on the adaptation of PLGA for the controlled release of antigen [47,48]. The entrapment of antigens in PLGA microspheres of 1–10 μm in diameter has been shown to enhance the immune response. The disadvantage of the PLGA system is the use of organic solvents and long preparation times for the microencapsulation of the antigens particularly labile antigens.

As mentioned above, the elucidation of a new type of ion cross-linkable synthetic polymer, PCPP has made it possible to generate microspheres containing antigens that throughout preparation are exposed only to an aqueous environment. Gelation by ionic crosslinking of an aqueous-based polymer solution at room temperature eliminates the long exposure to organic solvents, elevated temperatures and drying required by polymers dissolved in organic solvents.

PCPP can be combined with antigens in two different ways to effect immunopotentiation [32]. Antigens can be mixed with the soluble PCPP and injected directly into an animal for parenteral immunization. Alternatively, the water soluble PCPP and antigen solution can be formulated into hydrogel microspheres by cross-linking the carboxyl groups with divalent cations. These microspheres can then be used for parenteral or mucosal immunization.

4.1. PCPP as a vaccine adjuvant

In the soluble state, PCPP has demonstrated a remarkable adjuvant activity on the immunogenicity of formalin-inactivated influenza virions and commercial trivalent influenza vaccine [49]. Regardless of which antigen preparation was used, the addition of 100 μg of PCPP enhanced the functional HAI antibody response 10-fold over the levels elicited by the vaccine alone. Similarly, PCPP enhanced the IgM, IgG, and IgG1 ELISA antibody titers to influenza antigens at least 10-fold higher than the vaccine alone. Immunization of aged mice (22 months old) with trivalent influenza vaccine alone did not sero-convert these mice as measured by HAI or ELISA whereas significant sero-conversion was achieved when mice were immunized with PCPP formulated trivalent vaccine. The adjuvant activity of PCPP was shown to not be due to a site of injection depot effect. PCPP adjuvanticity was positively correlated to the molecular weight of the polymer.

The immunogenicity of antigens as diverse as tetanus toxoid, hepatitis B surface antigen, *Hemophilus influenzae* type b polyribosribotolphosphate, herpes simplex virus type 2 glycoprotein D [50] and HIV env [51] is dramatically enhanced in the presence of soluble PCPP. Except for the Hib polysaccharide antigen, a single dose of immunogen mixed with PCPP elicited a persistent high titer antibody response that lasted as long as 6 months. PCPP has, thus, proved to be a versatile and effective adjuvant. Furthermore, the data clearly showed that PCPP at very low concentrations is as efficient as complete Freund's adjuvant (CFA) in inducing high serum IgG responses to influenza and tetanus toxoid antigens. Perhaps more importantly, PCPP admixed with influenza antigens out performed CFA by eliciting high titer functional antibodies measured by hemagglutination inhibition and infectivity neutralization assays.

4.2. PCPP microspheres in vaccines

Conditions of encapsulation may play a very important role in maintaining immunogenic integrity of labile antigens. PCPP is a water-based ionicallycrosslinkable polymer that can be used to generate polymeric hydrogel microspheres. There are no organic solvents involved in the encapsulation procedure and therefore, labile immunological epitopes are most likely preserved. Mild encapsulation conditions, hydrophobic surface properties, ability to alter the side chains on the polymer and formulate microspheres that will release antigens with pulsatile and/or sustained release kinetics make this polymer system a strong candidate for developing single dose parenteral and mucosal vaccines.

PCPP microspheres have three desirable properties. Microspheres have the size and physical characteristics that facilitate uptake in the mucosal lymphoid tissue to stimulate an immune response. The microspheres are formulated under very mild conditions so that antigenic integrity is maintained. The hydrogel properties of these microspheres allow a sustained antigen release to maximally stimulate the immune response over a long period. The microsphere technology has demonstrated great utility in the microencapsulation of a diverse spectrum of biological materials.

The immunogenicity of influenza and tetanus toxoid was dramatically enhanced when microencapsulated in PCPP microparticles [32]. PCPP microencapsulated antigen was as efficient as complete Freund’s adjuvant in inducing high serum IgG responses. Furthermore, influenza encapsulated in PCPP microspheres elicited hemagglutination inhibition and neutralization antibody titers that were much higher than what was observed for alum and complete Freund’s adjuvant.

The high IgG and functional antibody titers elicited by PCPP microspheres could result from the sustained release of antigen from the microsphere matrix. In this mechanism, the matrix provides a superstructure that contains the protein antigens and then releases them through a combination of matrix erosion and antigen diffusion. This explanation ignores, however, the well established fact that the polymer molecules released through erosion would presumably have the adjuvant properties described for the soluble polymer. Obviously, the enhanced immune response could result from a combination of sustained release from the microsphere superstructure and the strong adjuvant property of resolubilized PCPP.

5. Conclusion

The multitude of possible structural manipulations combined with the relative ease of synthetic approaches in polyphosphazene chemistry continue to attract researchers tailoring polymer properties for specific biological applications. Biodegradable and hydrogel polyphosphazene matrices show tremendous potential as carriers for controlled release applications in in vitro and in vivo applications leaving ample room for polymer scientists to exercise their creativity in designing new protein delivery systems.

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