



SYNTHESIS OF COLON SPECIFIC N, N-BIS-(2-CHLOROETHYL) ANILINE POLYPHOSPHAZENE COPOLYMER CONJUGATES

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Received: 20 May 2010, Revised and Accepted: 24 Jun 2010

ABSTRACT

The aim of present study was to synthesize Polyphosphazene copolymers 4-amino-BCA conjugates containing an aromatic azo bond. The combination of colon specific aromatic azo bond cleavage resulted in a highly efficiently release of 4-amino-BCA from conjugate by caecal contents in vitro. The conjugates were also determined to be stable in simulated upper GI tract conditions. These Polyphosphazene copolymer BCA conjugate have the potential to enhance the therapeutic efficacy of 4-amino-BCA against colon cancer and to reduce side effects due to local drug release. Acidic PBS buffer, rat small intestine contents and small intestine mucosa solutions were used to simulate upper GI tract conditions, where rat caecum contents and large intestine mucosa solutions were used to simulate conditions in the colon. These experiments suggested that the Polyphosphazene copolymer- BCA conjugate containing an aromatic azo bond was stable in simulated upper GI tract conditions. Here, we reported the design and synthesis of Polyphosphazene co-polymer BCA conjugate, which are cleaved specifically in colon and produce 4-amino-BCA. Due to absence of peptide bond, this conjugate was not susceptible to attack by digestive enzymes in stomach and SI and demonstrated stability in acidic phosphate, SI mucosa and SI contents. Therefore it can be predicted that after oral administration, the conjugate will stay intact in upper GI tract. When the conjugate reaches the large intestine, its indigenous micro-flora degrades the azo bond and released 4-amino-BCA, remnants of linker group. Using this novel approach, Colon tumor cell may be effectively killed by high local drug concentration obtained by colon specific drug release

Keywords: 4-amino-BCA conjugates, Colon cancer, Colon specific drug release

INTRODUCTION

The anticancer drug N,N-Bis-(2-Chloroethyl)Aniline (BCA) is a synthetic nitrogen mustard derivative¹ and act by alkylating the N⁷ nitrogen of a guanine residue in one or both strands of DNA molecule.² This alkylating leads to cross-linkages between guanine residue in the DNA Chains and/or depurination that facilitates DNA strands breakage Fig.1.

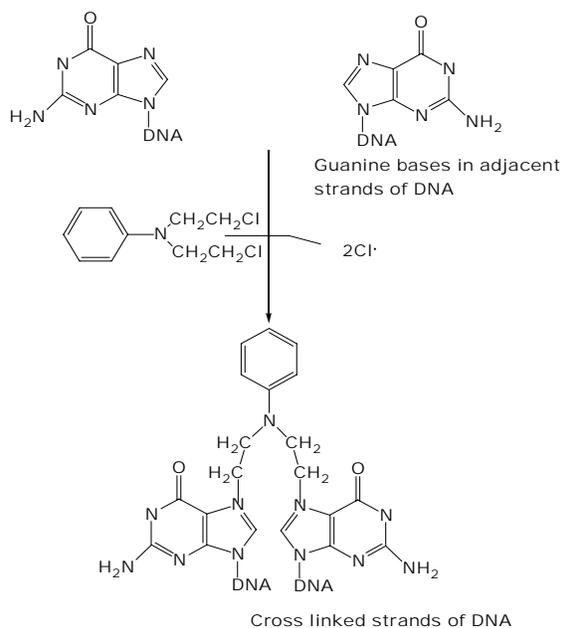


Fig. 1: Alkylation of guanine bases in DNA is responsible for the cytotoxic effect of BCA

But the BCA is less active against a wide spectrum of solid tumor including colon cancer. As G₁ and S phase specific drug, the drug requires prolonged exposure to the tumor with concentration exceeding a minimum threshold to achieve optimal therapeutic

efficacy.³ Water soluble Polyphosphazene⁴ Polymer might provide a platform to treat colon cancer by maintaining BCA concentration over its threshold in the colon by the use of colon specific drug delivery. Polyphosphazene polymer is bio-degradable polymer,^{5,6} where a drug is attached via a spacer that can be degraded at target site. To achieve colon-specific delivery, a BCA drug may be attached to Polyphosphazene copolymer side chains via aromatic azo bond cleavable by azoreductase activities present in the colon. e.g: release of 5-amino salicylic acid bound to HEMA copolymer via an aromatic azo bond was demonstrated using *streptococcus faecium*, an isolated strain of bacteria commonly present in colon,⁷ the caecum contents of rats, guinea pigs, rabbits⁸ and in human faeces.⁹ It was shown that the aromatic azo bond was cleaved in vitro¹⁰ resulting in the release of 4-amino-BCA.

A prodrug strategy was proposed that used to separate the drug by spacers which separated the enzymatically cleaved bond from the drug by a self eliminating group.¹¹⁻¹³ In this study, a novel colon specific BCA delivery system based on Polyphosphazene copolymer conjugates containing BCA and aromatic azo bond was designed and synthesized. Polyphosphazene copolymer BCA conjugates were prepared by radical copolymerization of Polyphosphazene with BCA. In vitro degradation of polymer conjugates by azoreductase activities in rat caecal contents was studied. The stability of conjugates in SI contents, SI mucosa, large intestine mucosa and in low pH and phosphate buffer was also investigated.

MATERIALS AND METHODS

Materials

BCA and Polyphosphazene were prepared as described below. All other chemicals were from Sigma Aldrich and Qualigens.

Synthesis

Poly(dichlorophosphazene), compound I

Hexachlorocyclotriphosphazene

Phosphorus pentachloride (50.0 g) was dissolved in chlorobenzene (250.0 ml) by slight heating. To this solution was added dry ammonium chloride (50.0 g), the reaction mixture was refluxed for 20 h. Outlet was connected to a trap containing 10% sodium hydroxide in order to neutralize the liberated hydrochloric acid. The

reaction mixture was allowed to cool to room temperature, filtered through glass wool to remove unreacted ammonium chloride and the solvent removed under reduced pressure to get the viscous oily residue. The residue was dissolved in pet. ether (60-80°C) (3 × 50.0 ml) to obtain the trimer and tetramer, concentrated (~50.0 ml) and extracted with concentrated sulphuric acid (3 × 15.0 ml) to get the cyclic trimer in acid layer. Sulphuric acid layer was diluted with water (~60%), the precipitated trimer extracted with pet. ether (60-80°C) (3 × 50.0 ml), refluxed with decolorizing carbon for 20 min, filtered, concentrated and kept overnight for crystallization. The monoclinic crystals so obtained were filtered, recrystallized from pet. ether (60-80°C) to get the pure trimer, (Yield: 7.6 g, 27.3%), m.p. 111-112 °C (Lit.¹⁴112°C).

Poly(dichlorophosphazene), compound I

The glass ampoule was cleaned with chromic acid for 1 day, washed with distilled water and dried in an oven at 110-120°C. Hexachlorocyclotriphosphazene so obtained (10.0 g) was taken in a cleaned, dried ampoule, evacuated and sealed under inert atmosphere. The ampoule was heated in a muffle furnace at 250 ± 10°C for 7 h. Thermal polymerization of the trimer gave transparent material, it was broken in the dry box, contents were carefully transferred to the sublimation apparatus in an inert atmosphere, sublimed at 80-90°C under vacuum to remove the unpolymerised trimer and pure poly(dichlorophosphazene) left behind was taken in the vial.

N, N-bis-(2-chloroethyl)aniline, compound II

N, N-bis-(2-hydroxyethyl)aniline

A mixture of aniline (4.5 ml, 48.6 mmol) and 2-chloroethanol (14.3 ml, 196 mmol) was added to a suspension of calcium carbonate (10.0 g in 250.0 ml of water). The cloudy mixture was refluxed for 24 h with stirring and additional 2-chloroethanol and calcium carbonate were added in two equivalent portions over the following 48 h. The reaction mixture was cooled, pH adjusted to pH 7 with sodium hydroxide solution (10%) and extracted with ethyl acetate (4 × 200.0 ml). The combined organic layer was dried and solvent removed under reduced pressure to get product bp 175-180° C (Lit.180°C).¹⁵

N, N-bis-(2-chloroethyl) aniline, compound II

N, N-Bis-(2-hydroxyethyl)aniline (2.7 ml, 14.9 mmol) in phosphorus oxychloride (9.0 ml) was refluxed for 40 min and hot reaction mixture was poured with stirring into crushed ice. The reaction mixture was extracted with ethyl acetate (4 × 100.0 ml) and washed with 10% sodium bicarbonate solution (3 × 200.0 ml). The combined organic layer was dried, filtered and solvent removed under reduced pressure to get the product; (2.28 g, 70.0%), mp 40-44°C (lit. 45°C)¹⁵.

4-(4-hydroxyphenylazo)-N, N-bis-(2-chloroethyl) aniline, compound III

Sodium nitrite solution (0.5 g in 2.0 ml water) was added with stirring at 5°C to the solution of 4-aminophenol (1.0 g, 8.1 mmol) in dilute sulphuric acid (10% v/v, 10.0 ml) kept at 5°C. To this was added solution of N,N-bis-(2-chloroethyl)aniline (2.2 g) in dilute sulphuric acid (10% v/v, 10.0 ml), the reaction mixture was stirred for 15 min at 0-5°C and basified with 10% sodium hydroxide. The precipitated material was filtered and dried to get the product; (1.6 g, 52.0%), mp 175-177°C.¹⁶

Poly[bis(4-(1-phenylazo)-N,N-bis-(2-chloroethyl)aniline)phenoxy]phosphazene, compound IV

Poly (dichlorophosphazene) (2.0 g, 17.0 mmol) was dissolved in dry tetrahydrofuran (50.0 ml) to yield clear viscous solution. A solution of Compound III (3.1 g, 34.0 mmol) in dry THF (50.0 ml) was added to it, followed by K₂CO₃ (2.0 g). The solution was refluxed for 90 h and filtered to remove KCl salts. The filtrate was concentrated under reduced pressure and purified by precipitation with petroleum ether (60-80°C) to obtain the compound IV, (1.5g, 76.0%).¹⁷

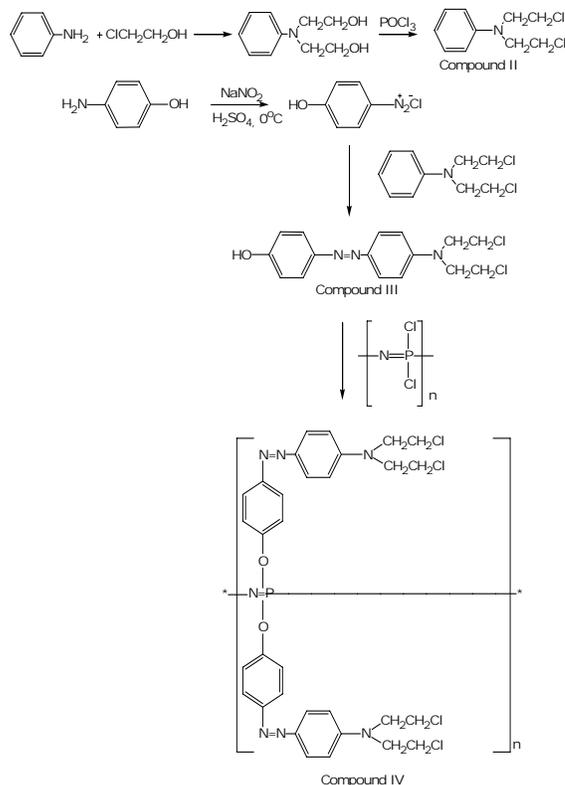


Fig. 2: Synthesis of Polyphosphazene BCA conjugates

In vitro drug release

Preparation of rat SI and caecal contents solution

SI and caecal contents were removed from wistar rats, suspended in cold PBS, pH 7.4. After homogenization and centrifugation of the suspension of SI contents, the supernatant was stored in refrigerator and used as SI contents. The suspension of caecal contents was bubbled with nitrogen and stored at -20°C.¹⁸

Preparation of rat SI and LI mucosa solutions

After washing the SI and LI with a cold saline solutions, the mucosa of SI and LI were scraped gently from the lumen of intestines using glass slides. The scraped mucosa was dispersed in cold PBS, pH 7.0, and homogenized using a blender. The final concentrations of the mucosa were 2% (w/v) after adjustment. All of mucosa suspensions were stored at -20°C.¹⁸

Release of nitrogen mustard from Polyphosphazene copolymer conjugates in rat caecal contents

The release of nitrogen mustard from polyphosphazene copolymer conjugates in rat caecal contents were investigated using UV method. The polyphosphazene nitrogen mustard copolymer conjugate was added to rat caecal content suspension under a nitrogen atmosphere. The final concentration of ceal contents was adjusted at 2% (w/v). Under anaerobic conditions, the mixture was incubated at 37°C and 0.5 ml samples were withdrawn at each scheduled time interval for 24 h study. All the samples were analysis by UV spectrophotometer at λ_{max} 351 nm.

Stability of Polyphosphazene-Nitrogen Mustard conjugates in SI contents, SI and LI mucosa solution and PBS buffer

To study the stability of Polyphosphazene-Nitrogen Mustard conjugates the polymer was incubated with the supernatant of SI contents, a 2% (w/v) SI mucosa suspension, a 2% (w/v) LI mucosa suspension, PBS buffer pH 1.5 and Cecal content (under anaerobic condition) at 37°C for 24 h. After incubation, 0.5 ml samples were

withdrawn from each incubation solution and analysis by UV spectrophotometer at specific wavelength.

RESULTS

Synthesis

The synthesis of BCA-containing monomer (compound-IV) is shown in Fig. 2. N,N-bis-(2-chloroethyl) aniline (Compound II) was synthesized by reaction of aniline and 2-chloroethanol which further reacted with diazonium chloride of 4-amino benzyl alcohol by coupling reaction in presence of nitrous acid to formed 4-(4-hydroxyphenylazo)-N,N-bis-(2-chloroethyl)aniline (compound III). The amino group of compound III reacted with polymer Polyphosphazene (compound I) to yield BCA-containing monomer (compound IV). The synthesized compounds were characterized by NMR. The assignment for compound IV is displayed in Table-1.

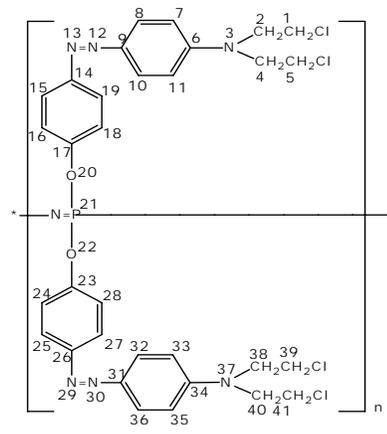


Table 1: NMR assignment for the BCA containing monomer

Atom no.	¹ H	Atom no.	¹ H	Atom no.	¹ H
1	3.87, t, 2H	15	7.77, d, 1H	29	(N=)
2	3.48, t, 2H	16	7.36, d, 1H	30	(=N)
3	(N)	17		31	
4	3.48, t, 2H	18	7.36, d, 1H	32	7.55, d, 1H
5	3.87, t, 2H	19	7.77, d, 1H	33	7.05, d, 1H
6		20	(O)	34	
7	7.05, d, 1H	21	(=P)	35	7.05, d, 1H
8	7.55, d, 1H	22	(O)	36	7.55, d, 1H
9		23		37	(N)
10	7.55, d, 1H	24	7.36, d, 1H	38	3.48, t, 2H
11	7.05, d, 1H	25	7.77, d, 1H	39	3.87, t, 2H
12	(=N)	26		40	3.48, t, 2H
13	(N=)	27	7.77, d, 1H	41	3.87, t, 2H
14		28	7.36, d, 1H		

Polyphosphazene BCA conjugates containing an aromatic azo bond were prepared by radical copolymerization of Polyphosphazene with BCA in THF using triethylamine (TEA).

In vitro BCA releases from Polyphosphazene copolymer conjugate

This experiment was designed to demonstrate that free drug was released efficiently in artificial conditions from Polyphosphazene copolymer BCA conjugate containing an aromatic azo bond. The drug release process from the conjugate is demonstrated in Fig. 3.

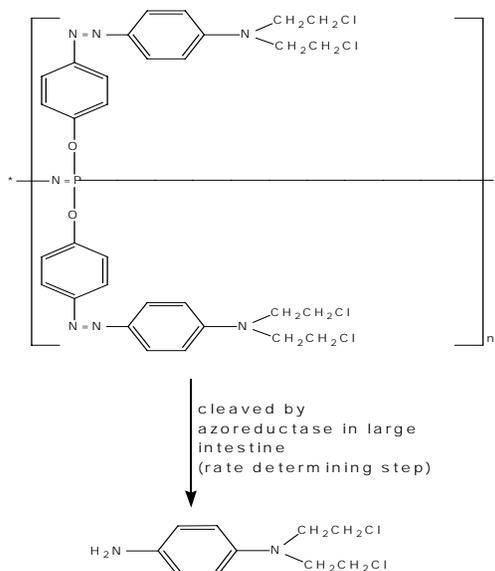


Fig. 3: Scheme of release of 4-amino-BCA from Polyphosphazene BCA copolymer conjugates by rate controlling aromatic azo bond cleavage

The cleavage of aromatic azo bond by azoreductase activities is the rate determining step. The study is done by using solution containing rat caecal contents with phosphate buffer in a nitrogen atmosphere to activate the bacterial enzyme. Drug release study initial carried out in 0.1 N HCl for 2 h followed by addition of 0.2 M trisodium orthophosphate, in order to obtain a final pH of 6.8 in the medium. The pH was adjusted with aid of 2 N HCl and 2 N NaOH. Samples were withdrawn at predetermined time intervals and replaced with fresh media. They were the analyzed using UV-spectrophotometer at λ_{max} 351 nm. After 5 h nitrogen was introduced into the medium in order to maintain conditions, at a rate which prevents the generation of turbulence in the dissolution medium. At the fifth hour fresh rat caecal contents (2% w/v) was also introduced into the dissolution medium. The samples are withdrawn at regular time interval for 24 h and analyzed with the aid of validated UV-spectrophotometer (λ_{max} 351 nm) (Fig. 4).¹⁹⁻²¹

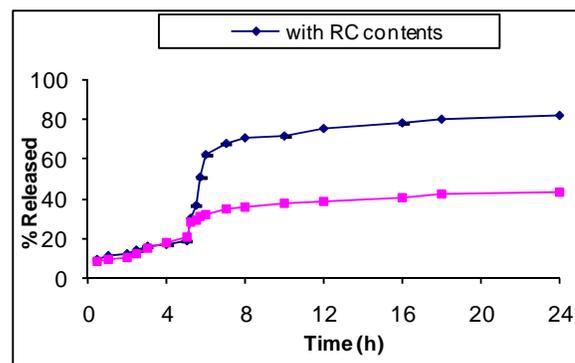


Fig. 4: Release of 4-amino-BCA from copolymer-BCA conjugates with or without rat caecal contents at 37°C.

Stability of conjugate in SI contents, SI mucosa, LI mucosa suspension and PBS buffer

Polyphosphazene copolymer BCA conjugate was incubated with SI content, SI mucosa, LI mucosa, PBS pH -1.5 and PBS pH-7.4

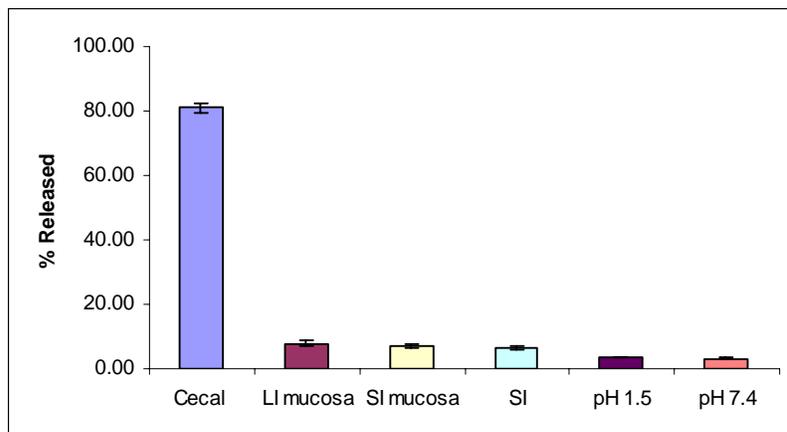


Fig. 5: Twelve hour cumulative release of 4-Amino-BCA from Polyphosphazene BCA conjugates during incubation with rat caecum contents, LI mucosa, SI contents, and PBS buffer, pH-1.5, pH-7.4 at 37°C.

No drug release was observed in PBS at pH 1.5 and 7.4 after 24 h of incubation, less than 4% drug release was detected after 24 h incubation with rat SI contents or SI mucosa and less than 6% drug release was measured after 24 h incubation with homogenized LI mucosa. Acidic PBS buffer, rat small intestine contents and small intestine mucosa solutions were used to simulate upper GI tract conditions, where rat caecum contents and large intestine mucosa solutions were used to simulate conditions in the colon. These experiments suggested that the Polyphosphazene copolymer- BCA conjugate containing an aromatic azo bond was stable in simulated upper GI tract conditions.

DISCUSSION

Polyphosphazene-BCA conjugate for the colon specific delivery of 4-amino-BCA were synthesized and evaluated in vitro studies. In these conjugate BCA was bound to polymer backbone with aromatic azo bond. Aromatic azo bonds were frequently used in the design of colon specific drug delivery systems. The bonds are cleavable by azoreductase activities present only in the colon. The reduction of azo bonds results in the formation of two amino groups. Various drug delivery based on azo groups in the main chain,²² side chains^{7,8} or in the cross links.²³⁻²⁵ The use of water-soluble co-polymers containing 5-amino salicylic acid (5-ASA) bound via an aromatic azo bond in side chains has been proposed by Brown et al.²⁶ such a design avoids drug absorption from SI with a concomitant decrease of side effects. The reduction of azo bond in this polymer conjugate released the 4-amino-BCA.

Here, we reported the design and synthesis of Polyphosphazene copolymer BCA conjugate, which are cleaved specifically in colon and produce 4-amino-BCA. The rate of azoreductase depends on the charge density of the azo region. A high electron density within the azo region is caused by the decrease of reduction of the azo bond to form aromatic amines via the hydro intermediates.²⁷ Due to absence of peptide bond, this conjugate was not susceptible to attack by digestive enzymes in stomach and SI and demonstrated stability in acidic phosphate, SI mucosa and SI contents (Fig. 5). It can be predicted that after oral administration, the conjugate will stay intact in upper GI tract. When the conjugate reaches the large intestine, its indigenous micro-flora degrades the azo bond and released 4-amino-BCA, remnants of linker group. Using this novel approach, Colon tumor cell may be effectively killed by high local drug concentration obtained by colon specific drug release.

respectively at 37°C for 24 h in a shaking water bath. The experimental conditions were selected to stimulate the GI tract to predict the behavior of conjugate during oral drug delivery. The release of 4-amino- BCA from the conjugate is summarized in Fig. 5.

CONCLUSION

Polyphosphazene copolymers 4-amino-BCA conjugates containing an aromatic azo bond were designed and synthesized. The combination of colon specific aromatic azo bond cleavage resulted in a highly efficiently release of 4-amino-BCA from conjugate by caecal contents in vitro. The conjugates were also determined to be stable in simulated upper GI tract conditions. These Polyphosphazene copolymer BCA conjugate have the potential to enhance the therapeutic efficacy of 4-amino-BCA against colon cancer and to reduce side effects due to local drug release.

REFERENCES

1. Turk JL. Studies on the mechanism of action of methotrexate and cyclophosphamide on contact sensitivity in the guinea pig. *Int. Arch. Allergy Appl. Immunol.* 1964; 24:191-200.
2. Evans R. Combination therapy by using cyclophosphamide and tumor-sensitized lymphocytes: a possible mechanism of action. *J. Immunol.* 1983;130:2511-2513.
3. Finch AR, Krishnamurthy S, Philip GP, Sartorelli CA. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino)carbonyl-hydrazine (101M): A Novel Sulfonylhydrazine Prodrug with Broad-Spectrum Antineoplastic Activity. *Cancer Research* 2001;61:3033-3038.
4. Allcock HR, Coley SM, Manners I, Visscher K, Parvez M. Reactivity and polymerization behavior of a pentachlorocyclocarbophosphazene, N3P2CCl5. *Inorg. Chem.* 1993;32 :5088-5094.
5. Manners I, Allcock HR, Renner G, Nuyken OJ. *J. Am. Chem. Soc. Poly(carbophosphazenes): a new class of inorganic-organic macromolecules.* 1989;111 :5478-5480.
6. Neilson RH, Hani R, Wisian-Neilson P, Meister JJ, Roy AK, Hagnauer GL. Synthesis and Characterization of Poly--(alkyl/arylphosphazenes). *Macromolecules* 1987;20:910-916.
7. Grim Y, Kopecek J. Bioadhesive water-soluble polymeric drug carriers for site-specific oral drug delivery. Synthesis, characterization and release of 5-aminosalicylic acid by *Streptococcus faeium* in vitro. *New Polym. Mater* 1991;3:49-59.
8. Kopeckova P, Rathi R, Takada S, Rihova B, Berenson MM, Kopecek J. Bioadhesive N-(2-Hydroxypropyl)methacrylamide copolymers for colon-specific drug delivery. *J. Control. Rel.* 1994;24:211-222.
9. Sakuma S, Lu ZR, Pecharova B, Kopeckova P, Kopecek J. n-(2-Hydroxypropyl)methacrylamide copolymer-9-aminocamptop-

- thecin conjugates: colon-specific delivery in rats. *J. Bioact. Compat. Polym.* 2002;17:305-319.
10. Sakuma S, Lu ZR, Kopeckova P. Biorecognizable HPMA copolymer-drug conjugates for colon-specific delivery of 9-aminocamptothecin. *J. Control. Rel.* 2001; 75:365-379.
 11. Carl PL, Chakravarty PK, Katzenellenbogen JA. A novel connector linkage applicable in prodrug design. *J. Med. Chem.* 1981;24: 479-480.
 12. Toki BE, Cerveny CG, Wahl AF, Senter PD. Protease-mediated fragmentation of p-amidobenzyl ethers: a new strategy for the activation of anticancer prodrugs. *J. Org. Chem.* 2002;67:1866-1872.
 13. De Groot FM, Loos WJ, Koekoedk R, van Berkom LW, Busscher GF, Seelen AE, Albrecht C, de Bruijn P, Scheeren HW. Elongated multiple electronic cascade and cyclization spacer systems in activatable anticancer prodrugs for enhanced drug release. *J. Org. Chem.* 2001;66:8815-8830.
 14. Allcock HR, Brennan DJ, Dunn BS. Synthesis of Polyphosphazene Bearing Geminal (Trimethylsilyl)methylene and alkyl or Phenyl Side Groups. *Macromolecules* 1989;22: 1534-1539.
 15. Newcomb PA, Orfleet RG, Storer BE, Surawicz TS, Marcus PM. Screening Sigmoidoscopy and Colorectal Cancer Mortality. *J. Nat. Cancer Inst.* 1992;84:1572-1575.
 16. Hardcastle JD, Chamberlain JO, Robinson NHE. Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. *Lancet* 1996;348:1472-1477.
 17. Allcock HR, Kugel RL. Phosphonitric Compounds. VII. High Molecular Weight Poly(diaminophosphazene). *Inorg. Chem.* 1966:1716-1718.
 18. Song QG, Zheng RL, Boris P, Pavla K, Jindrich K. Colon-specific 9-aminocamptothecin-HPMA copolymer conjugates containing a 1,6-elimination spacer. *J. Control Rel.* 2006;110:323-328.
 19. Mooter V, Samyn C, Kinget R. The relation between swelling properties and enzymatic degradation of azo polymers designed for colon-specific drug delivery. *Pharm Res.* 1994;11:1737-1741.
 20. Pongpaibul Y, Price JC, Whitworth CW. Preparation and evaluation of controlled release indomethacin microspheres. *Drug Dev Ind Pharm.* 1984; 410:1597-1616.
 21. Paharia A, Yadav AK, Jain SK, Rai G, Agrawal GP. Eudragit-coated Pectin Microspheres of 5-Fluorouracil for Colon Targeting. *AAPS Pharm. Sci. Tech.* 2007;8:E-1-5.
 22. Balasubramanian S, Kim J, Kim DY, Kumar J, Tripathy SK. Water-soluble, conjugated main chain azo polymer: synthesis and characterization, *Macromol. Rapid Commun.* 1996;17:853-857.
 23. Yeh PY, Kopeckova P, Kopecek J. Biodegradable and pH-sensitive hydrogels: synthesis by cross-linking of N,N-dimethacrylamide copolymer precursors. *J. Polym. Sci.* 1994;32: 1627-1637.
 24. Saffran M, Kumar GS, Savariar C, Burnham JC, Williams F, Neckers DC. A new approach to the oral administration of insulin and other peptide drugs. *Science* 1986;233:1081-1084.
 25. Van den Mooter G, Samyn C, Kinget R. Azo polymers for colon-specific drug delivery: II. Influence for the type of azo polymer on the degradation by intestinal microflora. *Int. J. Pharm.* 1993;97:133-139.
 26. Brown JP, McGarraugh GV, Parkison TM, Wingard JRE, Oderdonk AB. A polymeric drug for treatment of inflammatory bowel disease. *J. Med. Chem.* 1983;26:1300-1307.
 27. Brondsted H, Kopecek J. Hydrogels for site-specific drug delivery to colon: in vitro and in vivo degradation. *Pharm. Res.* 1992;9:1540-1545.