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Synthesis and Biological Properties of Polymer Immunoadjuvants

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ABSTRACT: New biodegradable and biocompatible polyphosphate based on L-Tyr–L-Tyr or L-Ser–L-Tyr dipeptide as the recurring units were synthesized. One of these polyphosphates was chosen for the study of adjuvanticity in conjunction with the soluble antigen extracted from adult *Schistosoma japonicum* (SjAg). The preliminary results show that, when measuring the serum antibody response to SjAg in female mice over 10 weeks, the polyphosphate (I) exhibited strong adjuvant activity.

KEY WORDS Polyphosphate / Immunoadjuvant / Synthesis / Tyrosine /

Modern synthetic, biochemical, and recombinant techniques have permitted the creation of increasingly pure polypeptide antigens to induce desired specific immune responses. But unfortunately, the small peptide antigens are generally weak immunogens. Therefore, the new generation of vaccines requires the use of strong adjuvants to raise the antibody responses to levels which will ensure protection against infectious disease.

The ideal adjuvant for human vaccines should be nontoxic, easily metabolized, stable in the absense of refrigeration and effective in accomplishing its desired goal. To date, there is no adjuvant that accomplishes all of these objectives. Of the adjuvants being studied, the majority of natural and synthetic adjuvants exhibit side effects. Only alum is licensed for use in human vaccine, but it is far from ideal. It may induce the formation of granuloma at the injection site and hypersensitive reactions. It is not always effective, increases cell-mediated immunity only slightly if at all, as it cannot be lyophilized, it requires refrigerated storage.[1]

It is known that the sustained release of antigen from a polymeric delivery device enhances its antibody response.[2] It would be expected that when an antigen is introduced into a biodegradable polymer system with adjuvant properties, the antibody responses could be much higher. Kohn *et al.*[3] first reported the synthesis and adjuvanticity of poly(Z–L-Tyr–L-Tyr–Hex-iminocarbonate), which was found to enhance the antigenicity of bovine serum albumin (BSA) in mice.

The fact that many hydrophobic and surface active substances can augment immune response[1] implies that compounds with hydrophobic or amphiphilic structures tend to have immunoadjuvanticity. In view of the known adjuvanticity of L-tyrosine derivatives[3] and good biocompatibility, biodegradability of polyphosphate,[4,5] we designed and synthesized a series of new polyphosphates with L-Tyr–L-Tyr or L-Ser–L-Tyr dipeptide as the recurring units. The chemical structures of these polyphosphates are shown in Figure 1. The

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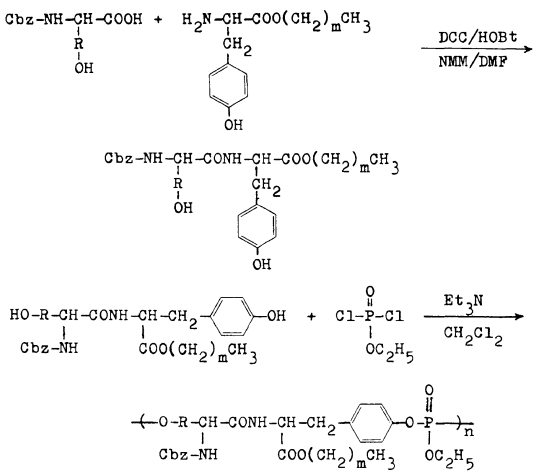


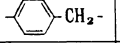
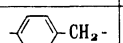
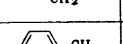
Dipeptide*	Polyphosphate	R	m
CTTH	I		5
CSTH	II	$-CH_2-$	5
CTTP	III		15
CSTP	IV	$-CH_2-$	15
CTTS	V		17
CSTS	VI	$-CH_2-$	17

Figure 1. Synthetic scheme of polyphosphates. *CTTP, *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine palmityl ester; CSTP, *N*-benzyloxycarbonyl-L-seryl-L-tyrosine palmityl ester; CTTS, *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine stearyl ester; CSTS, *N*-benzyloxycarbonyl-L-seryl-L-tyrosine stearyl ester.

stearyl or palmityl side chain will make the polyphosphates more hydrophobic, and the incorporation of serine residue into the polymer backbone chain will increase the hydrophilicity of the backbone. One of these polyphosphates has been used for the study of adjuvanticity in conjunction with the soluble antigen extracted from adult *Schistosoma japonicum* (SjAg).

EXPERIMENTAL

Synthesis and Characterization

Materials and Apparatus. Tyrosine hexyl ester hydrochloride (Tyr–Hex–HCl), *N*-hydroxybenzotriazole (HOBt), and ethyl phosphodichloridate were prepared according to literature 6, 7, and 8, respectively. Triethylamine (Et_3N), *N*-methylmorphorine (NMM), dichloromethane, and dimethylformamide (DMF) were dried and redistilled before use. Dicyclohexylcarbodiimide (DCC) was purified by redistillation.

Gel permeation chromatography (GPC) was performed on a Shimadzu LC-4A high pressure liquid chromatography (HPLC) system. Tetrahydrofuran (THF) was used to elute the sample through HSG20 polystyrene column (10 μm) (1 ml min^{-1}, 25°C). The detection was done at 280 nm (UV detector). The system was calibrated with monodispersive polystyrene standards. Fourier transform infrared spectroscopy (FT-IR) was performed on a Nicolet 170SX machine (KBr plate). Proton nuclear magnetic resonance spectra were recorded on a FX-90Q instrument (90 MHz, in deuterated chloroform). Elemental analyses were determined on Carlo Erba 1106 model instrument.

Synthesis of Dipeptide Monomers. To a cold solution (0°C) of *N*-Cbz–Ser (2.39 g, 10 mmol), Tyr–Hex–HCl (3.02 g, 10 mmol), HOBt (3.06 g, 20 mmol) and NMM (1.1 ml, 10 mmol) in 45 ml DMF, DCC (2.27 g, 11 mmol) was added. The reaction mixture was stirred for 24 h at room temperature, then filtered, the filtrate was evaporated to dryness, the residue was redissolved in EtAc, washed with 1 *N* HCl (50 ml × 3), 1 *N* NH_4OH (50 ml × 3), saturated NaCl solution (50 ml × 3), and dried with anhydrous Na_2SO_4, evaporated to dryness, the crude product was purified by flash chromatography using EtAc–MeOH–hexane (20 : 1 : 10) as eluent, then was recrystallized twice, *N*-Benzyloxycarbonyl-L-seryl-L-tyrosine hexyl ester (CSTH) was obtained as white powder, yield 3.5 g (72%). The other five dipeptides obtained as white powders were prepared according to the same procedure (Figure 1).

Synthesis of the Polyphosphates. To a cold

solution (0°C) of *N*-Benzyloxycarbonyl-L-tyrosyl-L-tyrosine hexyl ester (CTTH) (1.125 g, 2 mmol) and triethylamine (0.56 ml, 4 mmol) in 15 ml CH_2Cl_2, ethyl phosphodichloridate (0.326 g, 2 mmol) in 10 ml CH_2Cl_2 was added dropwise, the reaction was stirred for 24 h at room temperature. The solution was washed with saturated NaCl solution (20 ml × 3), dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in methanol and reprecipitated in ether, the polyphosphate (I) was dried under vaccum, and obtained as yellowish powder, yield 0.82 g (63%).

By the same method, polyphosphates (II)—(VI) were obtained.

In Vitro Degradation Study of the Polyphosphates

In Different pH Media. In vitro degradation studies were carried out by adding 100 mg of the samples in 10 ml distilled water which was adjusted to a given pH with dilute NaOH or HCl solution. The pH values of the suspension were recorded on PH/ISE acidimeter (ORION SA720) at regular intervals (20°C).

In Phosphate Buffered Saline (PBS). The tests were conducted by placing disc samples in 20 ml 0.1 M pH 7.4 PBS at 37°C. The disc samples were fabricated by compression molding at room temperature and a pressure of 400 MPa. The discs were 11 mm in diameter, approximate 1.5 mm in thickness, and about 200 mg in weight. The samples were removed after 10 days, dried to constant weight under vaccum, and analyzed for weight loss.

Determination of the Biological Activities

Materials. 4—5 week-old female Kunming mice were used. The soluble antigen from adult *Schistosoma japonicum* (SjAg) and complete Freund's adjuvant (CFA) were offered by Hubei Medical University.

Preparation of the complex of the polyphosphate (I) and SjAg. Particles of polyphosphate (I) with an average diameter of 10 μm (made by colloidal mill) were dispersed in 0.1 M PBS (pH 7.0), and the adjuvant suspension was sterilized in the autoclave at 116°C for 15 min and stored at 4°C. The complex of polyphosphate (I) and SjAg was prepared by mixing the adjuvant suspension and SjAg solution, the concentration of polyphosphate (I) and SjAg were adjusted to 5 mg ml^{-1} and 100 μg ml^{-1}, respectively. The complex was stirred for 24 h at room temperature, and used to immunize the mice directly.

Immunization and Measurement of Antibodies. The animals were randomly divided into 3 groups, 25 animals in each group. Animals in groups A—C received a subcutaneous, primary injection on day 0, followed by two booster injections on days 14 and 21. The primary injections were 50 μg SjAg in 0.1 ml normal saline (NS) (group A, as negative control), 50 μg SjAg in 0.1 ml CFA (group B, as positive control), 50 μg SjAg + 2.5 mg polyphosphate (I) in 0.5 ml PBS (group C). The booster injections were 20 μg SjAg in 0.1 ml NS (group A), 20 μg SjAg in 0.1 ml CFA (group B), 20 μg SjAg + 1 mg polyphosphate (I) in 0.2 ml PBS (group C).

Three animals in each group were bled every week, the serum samples were separated and combined, and stored at refrigerator (−20°C). The antibody titers were measured by enzyme-linked immunosorbent assay (ELISA).

RESULTS AND DISCUSSION

Synthesis and Characterization of the Polyphosphates

The dipeptide monomers were prepared by the carbodiimide coupling method following the standard procedures of peptide chemistry.[9] Their structures were ascertained by FT-IR, ^{1}H NMR, and elemental analysis (Tables I and II).

Polyphosphates were obtained by solution polycondensation using triethylamine as acid acceptor in dichloromethane at room temperature. The resulting polymers are slightly yellowish powder or wax-like solid, and have

Table I. Experimental data on dipeptide monomers

Compound	Yield	mp	Elemental analysis/%[b]		
	%	°C	C	H	N
CTTH	65	154—155	68.30	6.99	4.95
		(154—156)[a]	(68.31)	(6.81)	(4.98)
CSTH	72	107—108	63.87	7.12	5.58
			(64.18)	(7.08)	(5.76)
CTTP	69	125—126	71.51	8.48	3.93
			(71.76)	(8.32)	(3.99)
CSTP	75	101—103	69.25	8.40	4.33
			(68.95)	(8.68)	(4.47)
CTTS	73	126—128	71.89	8.05	3.26
			(72.29)	(8.55)	(3.83)
CSTS	78	105—107	69.97	8.96	3.93
			(69.69)	(8.93)	(4.28)

[a] mp reported in ref 5.
[b] Figures in parentheses show calculated data.

Table II. Spectra of dipeptide monomers[a]

Compound	^{1}H NMR (δ/ppm, DMSO-d_6)	FT-IR ($\tilde{\nu}$/cm^{-1}, KBr plate)
CTTS	0.9 (3H, CH_3) 1.0—1.6 (32H, $(CH_2)_{16}$) 2.8—3.4 (4H, –CH_2–Ph) 3.8—4.4 (4H, –CH_2–O, –N–CH–CO–) 4.9 (2H, –Ph–CH_2–O–) 6.5—7.4 (15H, Ph–H, Ph–OH) 9.1, 8.2 (2H, –CONH–)	3478, 3300 (OH, NH) 2919, 2850 (CH_3, CH_2) 1733, 1697, 1652 (–COO–, –CONH–) 1614, 1536, 1516, 1440 (–CONH–, –C–N–, –Ph–) 1268, 1234, 1206, 1085 (–C–O–)
CSTS	0.9 (3H, CH_3) 1.0—1.7 (32H, $(CH_2)_{16}$) 2.8—3.4 (2H, –CH_2–Ph) 3.6—4.3 (6H, –CH_2–O, –N–CH–CO–) 5.0 (2H, Ph–CH_2–O–) 6.5—7.4 (11H, Ph–H, Ph–OH) 9.1, 8.2 (2H, –CONH–)	3447, 3260 (OH, NH) 2919, 2850 (CH_3, CH_2) 1738, 1691, 1657 (–COO–, –CONH–) 1538, 1517, 1470 (–CONH–, –C–N–, –Ph–) 1261, 1243, 1211, 1045 (–C–O–)

[a] As representative examples, the ^{1}H NMR and FT-IR spectra of CTTS and CSTS are listed here.

good solubility in methanol, THF, DMSO, and dichloromethane, but are insoluble in water, ether, hydrocarbons. Polyphosphate (II), (IV), and (VI) which contain serine residue were found to be more hygroscopic than polyphosphate (I), (III), and (V), respectively.

The structures of the polyphosphates were confirmed by FT-IR, ^{1}H NMR, and elemental analysis. The experimental data are listed in Tables III and IV.

Polymer Degradation in Vitro

The polymers may be degraded in the presence of a nucleophile, such as the hydroxyl ion. Theoretically, water should cleave the phosphoester bond, yielding the dipeptide, alkanol, and phosphoric acid, as shown in eq 1.

Table III. Experimental data on polyphosphates

Polyphosphate	Appearance	Yield	$\bar{M}_w$ ($\times 10^4$)	Elemental analysis (%)[a]		
		%		C	H	N
I	Yellowish powder	63	1.89	61.51	6.46	4.59
				(62.56)	(6.35)	(4.29)
II	Yellowish powder	51	1.28	57.02	6.95	4.62
				(58.32)	(6.47)	(4.86)
III	Yellowish powder	71	0.91	65.85	7.88	3.41
				(66.64)	(7.75)	(3.53)
IV	Yellowish wax-like solid	75	1.31	62.11	8.60	3.57
				(63.67)	(8.02)	(3.91)
V	Yellowish powder	68	1.45	66.55	8.22	3.33
				(67.29)	(7.98)	(3.41)
VI	Yellowish wax-like solid	76	1.01	62.69	8.56	3.06
				(64.49)	(8.25)	(3.76)

[a] Parentheses show calculated data of elemental analysis.

Table IV. Spectra of polyphosphates[a]

Polyphosphate	^{1}H NMR (δ/ppm, $CDCl_3$)	FT-IR ($\bar{\nu}$/cm^{-1}, KBr plate)
I	0.9 (3H, CH_3) 1.0—1.4 (11H, CH_3, $(CH_2)_4$) 2.8—3,2 (4H, $-CH_2-Ph$) 3.6—4.4 (6H, $-CH_2-O$, –N–CH–CO) 5.2 (2H, Ph–CH_2–) 6.6—7.4 (15H, Ph–H, –CO–N–)	3302 (b, –NH–) 2952, 2932, 2859 (CH_3, CH_2) 1723, 1679 (–COO–, –CONH–) 1230, 1216 (b, P=O, –C–O–) 1088, (P–O–C)
II	0.9 (3H, CH_3) 1.0—1.4 (11H, CH_3, $(CH_2)_4$) 2.8—3.2 (2H, $-CH_2-Ph$) 3.6—4.8 (8H, $-CH_2-O$, –N–CH–CO) 5.0 (2H, Ph–CH_2–) 6.6—7.4 (11H, Ph–H, –CO–NH–)	3330 (b, –NH–) 2957, 2932, 2859 (CH_3, CH_2) 1732, 1680 (–COO–, –CONH–) 1235 (b, P=O, –C–O–) 1093 (P–O–C)

[a] As representative examples, the ^{1}H NMR and FT-IR spectra of polyphosphate (I) and (II) are listed.

$$-(-O-R^1-O-\underset{\underset{OR^2}{|}}{\overset{\overset{O}{\|}}{P}}-)- + H_2O \rightleftharpoons HO-R^1-OH + R^2OH + H_3PO_4 \quad (1)$$

As a representative example, a degradation study on polyphosphate (I) in different media is shown in Figure 2. As expected, hydrolysis was base catalyzed. The degradation rate is considerably faster in alkali medium than that in neutral or acidic medium.

The rate of degradation could be influenced by the character of the side chain and hydrophilicity of the polymer backbone. The results of *in vitro* degradation studies conducted in pH 7.4 PBS (0.1 M) at 37°C are shown in Figure 3. The polyphosphates with a L-Ser–L-Tyr recurring unit degraded much faster. The polyphosphate (II), (IV), (VI), and (I) swelled quickly, and changed dramatically in shape. The disc of polyphosphate (II), the most hydrophilic, nearly disappeared on the third day. It is concluded from Figure 3 that greater cleavage of the phosphoester bonds occurrs in

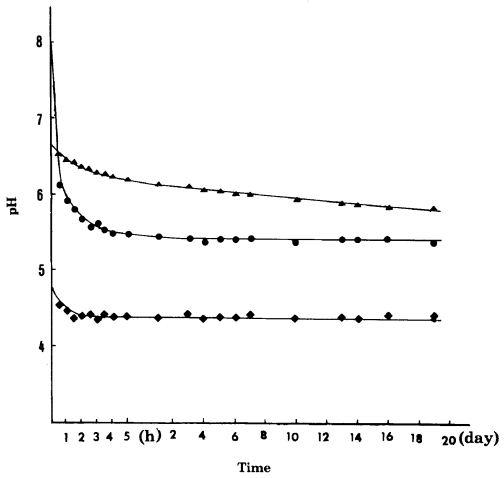


Figure 2. *In vitro* degradation of polyphosphate (I) in different pH medium.

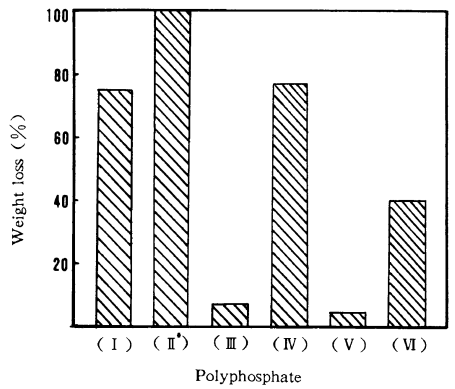


Figure 3. *In vitro* degradation of polyphosphates (I)—(VI) in PBS (0.1 M, pH 7.4, 37°C). *The weight loss of polyphosphate (II) is estimated to be 100% by the first three days.

polymers where water penetrates more readily, and the polyphosphates with long alkyl chains are more stable to cleavage and degradation.

Biological Study

The soluble antigen extracted from adult *Schistosoma japonicum* (SjAg) was chosen for testing adjuvanticity. Antibody response was measured as antibody titers to SjAg in female mice over 10 weeks. Figure 4 shows the antibody titers for each of the experimental groups.

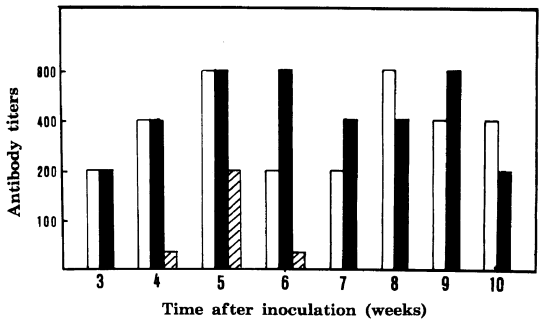


Figure 4. Antibody titers as determined by ELISA. □ Group C, SjAg complexed with polyphosphate (I); ■ Group B, SjAg in CFA (as positive control); ▨ Group A, SjAg in NS (as negative control).

It is evident that, SjAg complexed with polyphosphate (I), results in as a high antibody response as that of SjAg in complete Freund's adjuvant (CFA).

In conclusion, new biodegradable and biocompatible polyphosphates containing L-Tyr–L-Tyr or L-Ser–L-Tyr dipeptide were synthesized by solution polycondensation. A preliminary biological test indicated the polyphosphate (I) to possess strong adjuvant activity.

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