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FORMULATION AND CHARACTERIZATION OF MAGNETIC POLYGLUTARALDEHYDE NANOPARTICLES AS CARRIERS FOR POLY-l-LYSINE-METHOTREXATE

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Abstract

Using the statistically optimised method, submicron magnetic polyglutaraldehyde nanoparticles (Fe-PGNP) with free surface carboxylic groups have been synthesized. A model anticancer agent methotrexate (MTX) has been chemically bonded onto the surface of these particles using poly-l-Lysine (PL) as a spacer. The drug release characteristics of the final delivery device, i.e. [Fe-PGNP]-PL-MTX, has been elucidated at 37°C in a medium containing a proteolytic enzyme. Results demonstrate that using particles containing about 8% w/w of Fe_3O_4, and PL-MTX

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conjugate constituting 256 μg MTX per mg of PL, almost 50% of the conjugate can be covalently linked onto the carrier surface. Release studies failed to demonstrate the presence of free drug. However it appears that MTX-oligopeptides are released from the carrier as a result of the enzymatic hydrolysis of biodegradable bonds. It is suggested that [Fe-PGNP]-PL-MTX may be useful in the intracellular active targeting of bonded drug(s).

Introduction

Several workers have proposed the application of polyglutaraldehyde nanoparticles (PGNP) in cell-labeling, hemoperfusion and drug delivery (1-3). Following the inclusion of magnetite (Fe_3O_4), and intra-arterial administration of these particles in presence of a suitable magnetic field, PGNP can be easily retained at a desired target-site without being cleared by the reticuloendothelial system. This may allow PGNP to behave as an active drug delivery device (4,5). If some drug or drug-conjugate is linked onto the surface of these particles via a enzyme-labile or pH-sensitive bond, PGNP may offer third-order active drug targeting (6). PGNP are known to possess free surface carboxylic groups which may be used to link a drug or drug-conjugate containing free amino group(s) (5,6). Linkage of a drug directly on to the surface of PGNP may result in the formation of stable amide bonds which will prevent drug release at the target site (1,3). In the present study, a cytotoxic drug has been covalently linked on to the surface of magnetic polyglutaraldehyde nanoparticles (Fe-PGNP) in such a manner that intracellular or lysosomal enzymes are required for the release of drug (7,10).

Poly-l-Lysine (PL) is a polyamino acid which has been investigated in the past for the delivery of enzymes, drugs and growth inhibitory factors (7,11-14). This carrier has been demonstrated to act as a lysosomotropic agent for the delivery of methotrexate (MTX) (7,15). Conjugation of albumin or horseradish peroxidase with PL have been shown to cause several hundred fold increase in their cellular uptake

(11,12). In addition, the l-isomer of PL is biodegradable (8), and it increases the cytotoxic activity of MTX in cells normally resistant to this drug (7,12). The adsorption of PL onto polystyrene beads has been shown to enhance the phagocytosis of the particles (16). Arnold et al. (14) have shown that following the conjugation of a growth inhibitory derivative of nicotinamide to PL, the therapeutic effectiveness of the derivative is considerably improved. Of particular interest is the previous finding that the degradation products of PL-MTX are cytotoxic to cells in vitro (13), and hence free MTX is not essential for its action. For these reasons PL has been used as a biodegradable spacer to link MTX on to the surface of Fe-PGNP in this study. The inclusion of PL between the Fe-PGNP and MTX is envisaged to permit intracellular action of drug, and thus ensure third-order drug delivery.

Materials and Methods

Apparatus and Materials

The apparatus and materials used for the synthesis and characterization of Fe-PGNP were similar to that described previously (6). The chromatographic system for the quantitation of MTX was identical to that employed by Hung et al. (17). Absorption spectra and gel chromatographic measurements were obtained on a Shimadzu UV-240 spectrophotometer. The iron content of the particles was determined using a Shimadzu atomic absorption spectrophotometer (AA) equipped with a background correction.

Ferrofluid (30% w/w Fe_3O_4 in water) was obtained from Ferrofluidics Corp. (Nashua, New Hampshire, USA). MTX (USP), Lot X3100, was a gift from Lederle Laboratories Division, American Cyanamide Co. (Pearl River, NY) and Folic acid,USP (FA) was purchased from Fluka AG (Buchs SG, Switzerland). N-(3-dimethyl aminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC), disodium hydrogen phosphate, trisodium citrate and citric acid were obtained from BDH. Poly-l-Lysine hydrobromide (average MW 60,000) and Pancreatic

Crude Protease were purchased from Sigma Chemical Co. (St. Louis, Mo). Sephadex G-25 fine and G-50 fine from Pharmacia (Uppsala, Sweden) were used for gel chromatography. Phosphoric acid and Salicylaldehyde (SA) were purchased from May & Baker and HPLC grade methanol from Ajax Chemicals (Australia). All reagents were of AnalaR or equivalent grade and water was double distilled and MilliQ® filtered. All glassware were silanised using Aquasil® from Pierce Chemical Co. (Rockford, IL).

Synthesis and analysis of Fe-PGNP

The method previously optimised for the production of PGNP (6) was used as the basis of synthesizing Fe-PGNP. A 2.2% w/v of ferrofluid was added to the reaction mixture before the initiation of the polymerization. After the collection of magnetic particles, the unincorporated magnetite was removed by the method reported previously (18). The size, polydispersity, surface carboxylic group content and the magnetite content of Fe-PGNP were determined using the methods similar to that employed earlier (6,18).

Chromatography

The mobile phase consisted of 25% v/v methanol in aqueous buffer containing 10 mM trisodium citrate and adjusted to pH 3.5 with 5M citric acid. A flow rate of 2 ml/min and a detection wavelength of 305 nm were used. Salicylaldehyde (4 μg/ml) (SA) was used as an internal standard.

Synthesis of N^{10} - methyl folic acid (N^{10}-mFA) - Due to unavailability of N^{10}-mFA from the commercial sources, this compound was synthesized using the method similar to that reported by Hansen et al. (19), and used as a reference standard in the development of HPLC assay for MTX and its decomposition products.

Synthesis of PL-MTX

Twenty five mg of MTX was dissolved in 5 ml of 100 mM phosphate buffer (pH 7.9). Fifty mg of PL and 75 mg of EDC were

Table 1

The amounts of Fe-PGNP, PL-MTX and EDC used in the synthesis of [Fe-PGNP]-PL-MTX, and their efficiency in the covalent linkage of PL-MTX on to Fe-PGNP.

Exp. No.	Fe-PGNP (mg)	PL-MTX (mg)	EDC (mg)	% PL-MTX associated	µg PL-MTX per mg Fe-PGNP
1	10	0.8	50	50	40
2	10	1.4	50	45	63
3	10	2.0	50	40	80
4	10	2.0	50	41	82
5	10	2.6	50	36	94

added and the mixture kept in dark for about 2 hr with frequent shaking. In order to dissolve the insoluble PL-MTX, a small aliquot of 9M phosphoric acid was added and the resulting solution subjected to gel separation (12).

A Sephadex G-50 column, 86 x 24 mm i.d., with a total volume of 46 ml, was equilibrated with 100 mM phosphate buffer (pH 2.5). Following the addition of PL-MTX onto the column, 2.8 ml fractions of the eluant were collected per minute and their absorbance recorded at 305 nm. The fraction of eluant between 39.2 to 70.2 ml was analysed chromatographically for the presence of free MTX, and hence used for the determination of the amount of drug coupled to PL.

Linkage of PL-MTX onto Fe-PGNP

To 4 ml of 100 mM phosphate buffer (pH 7.0), containing 50 mg of EDC and a known amount of PL-MTX (see Table 1), 10 mg of

Fe-PGNP were suspended. The suspension was shaken gently in dark for 2 hr. The particles were then separated from the unbound PL-MTX by washing four times with water, each time centrifugating at 2000 xg for 10 min.

Enzymatic hydrolysis of PL-MTX and [Fe-PGNP]-PL-MTX

To 500 µl of phosphate buffer (pH 7.8) containing 0.15 mg of pancreatic crude protease, 1 mg of PL-MTX was added and the contents incubated for about 4 hr at 37°C (7,12). After the completion of incubation, 100 µl of 9M phosphoric acid was added and the solution injected onto a Sephadex G-25 gel chromatographic column, 120 x 15 mm i.d., with a total volume of 23 ml. Eluant fractions of 3.5 ml were collected per minute and their absorbance recorded at 305 nm. The eluant fraction between 17.5 to 21 ml was analysed chromatographically for the presence of free MTX.

In order to elucidate the enzymatic hydrolysis of [Fe-PGNP]-PL-MTX, 10 mg of this drug carrier was incubated in the medium as mentioned above. Before gel separation, the particles were isolated from the hydrolytic medium by centrifugation at 2000 xg for 10 min.

Results and Discussion

Characteristics of Fe-PGNP

The Fe-PGNP synthesized in this study had a mean diameter < 1 µm (see Fig. 1). Their polydispersity and surface carboxyl group concentration varied between 30 to 55% and 50 to 250 x 10^{-7} moles/gm, respectively. The particles contained 7.9 ± 1.2% w/w of Fe_3O_4. Previous work on the synthesis of Fe-PGNP has shown that addition of 1 to 5% w/v of ferrofluid to the initial reaction mixture of polyglutaraldehyde produced particles with a maximum of 14% w/w of Fe_3O_4 (5). Preliminary experiments carried out as a part of the present study however revealed that the addition of more than 2.2% w/v of ferrofluid did not improve the entrapment of magnetite. Hence 2.2% w/v of ferrofluid was used during the formulation of Fe-PGNP.

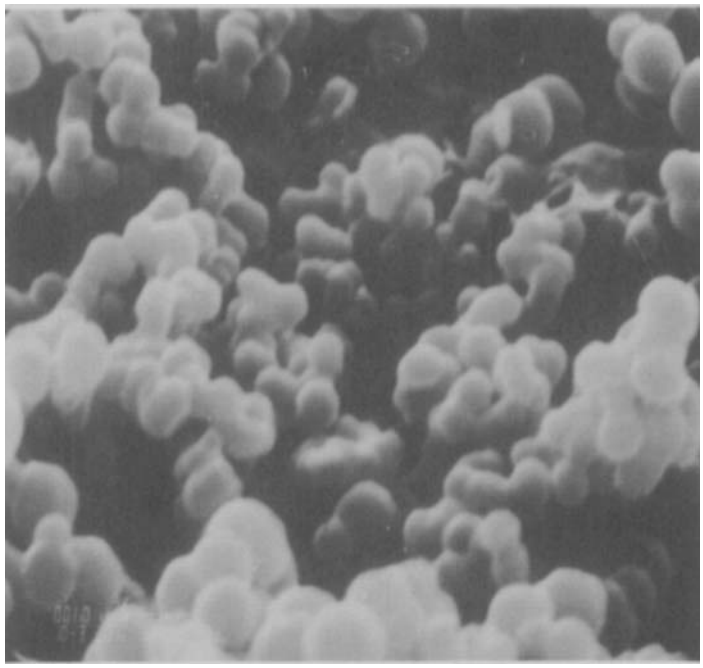

Figure 1

A representative photograph of Fe-PGNP. Note that the size of the particles is < 1 μm.

Chromatography

Fig. 2 shows a representative chromatogram of the four compounds under the optimised conditions. The retention times for FA, N^{10}-mFA, MTX and SA were 1.9, 3.2, 4.5 and 6.0 min, respectively. The within and between day variability of this assay was ≤ 5.4%.

Synthesis of PL-MTX and [Fe-PGNP]-PL-MTX

PL was initially conjugated to MTX and the resulting product was then covalently bonded onto Fe-PGNP. This covalent bonding reaction was catalysed by the presence of EDC (12). Figure 3 shows a gel separation chromatogram for PL-MTX. Two major peaks were eluted, the

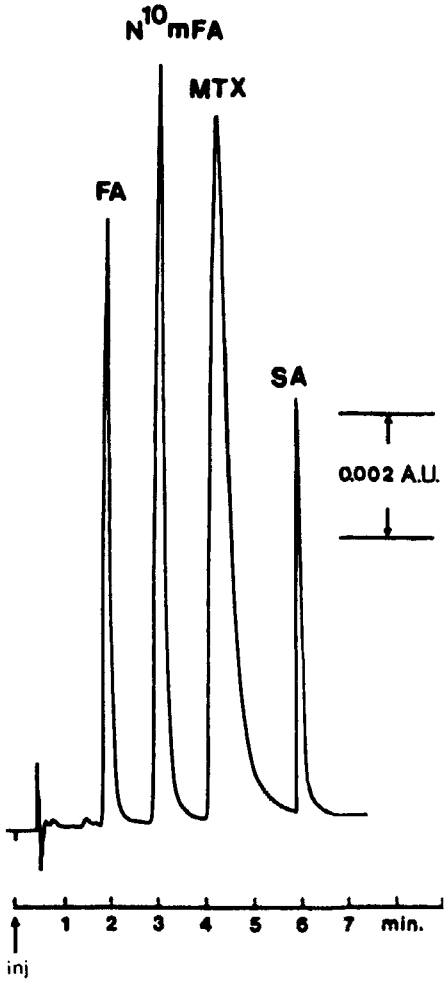

Figure 2

A representative HPLC chromatogram for MTX (200 ng), FA (50 ng), N^{10}-mFA (125 ng) and SA (80 ng) under optimal conditions. Refer Materials and Methods section for the chromatographic conditions.

first one being due to PL-MTX. Using HPLC it was confirmed that the second peak was due to free MTX. The eluant fractions comprising of second peak were pooled and their MTX concentration determined. It was found that 12.82 mg of MTX reacted with PL, resulting into a conjugate with 256 µg MTX per mg of PL. On molar basis, this equals 30 mol

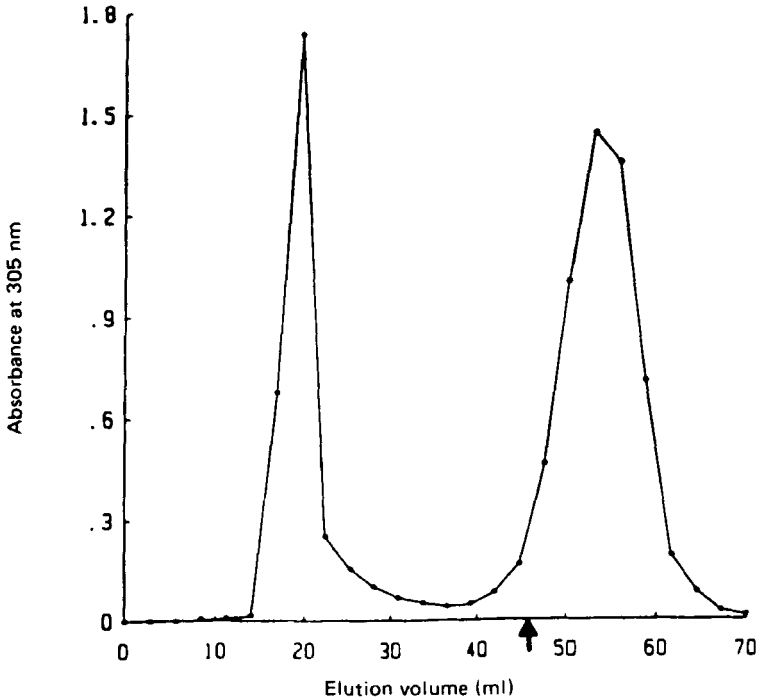

Figure 3

A gel separation chromatogram for PL-MTX and free MTX. Arrow represents the column volume. Refer Materials and Methods section for the gel chromatographic conditions.

MTX per mol of PL. This also indicates that approximately 1 in 10 lysine residues were linked to MTX, which is higher than that reported previously (1 in 27) (7).

Following the equilibration of PL-MTX with Fe-PGNP in the presence of EDC for 2 hr, amide bonds were formed between the free carboxylic groups on the surface of Fe-PGNP and the free amino groups of PL-MTX. Table 1 shows the percentage and absolute amounts of PL-MTX bonded onto Fe-PGNP surface. Good linearity ($r^2 > 0.98$) was observed between the amount of PL-MTX used and its amount bonded on

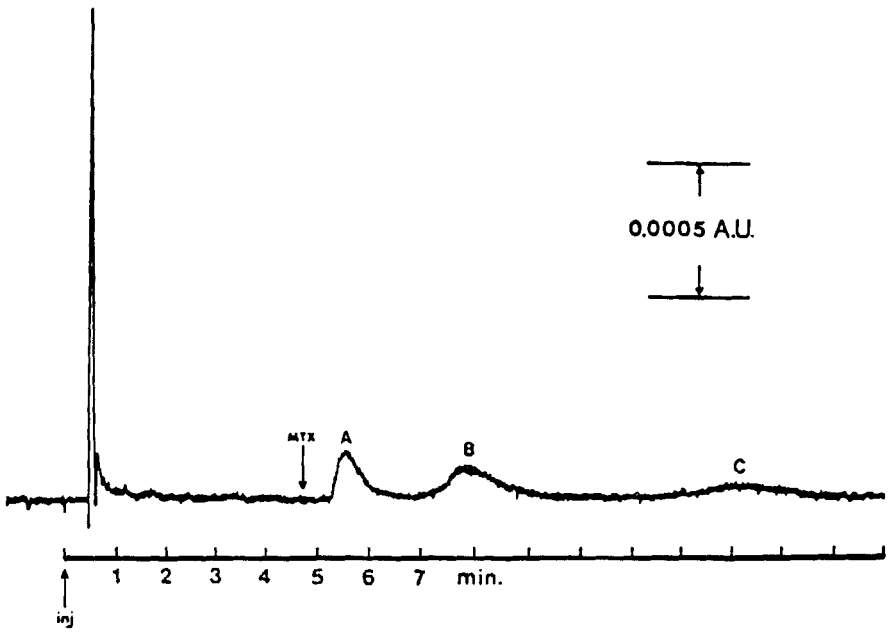

Figure 4

An HPLC chromatogram for the enzymatic hydrolytic products of [Fe-PGNP]-PL-MTX. Note the absence of MTX peak at about 4.5 min, but presence of peaks A, B and C.

Fe-PGNP. The minimum and maximum amounts of conjugate linked varied between 40 to 94 μg per mg of Fe-PGNP.

Enzymatic hydrolysis

PL is a polypeptide which is readily hydrolysed by proteolytic enzymes into various oligopeptides (12,13). Shen and Ryser (13) have established a close correlation between the products of the in vitro incubation of PL-MTX and that observed in cell cultures. Hence in order to confirm the enzymatic lysis of PL-MTX and [Fe-PGNP]-PL-MTX, these products were incubated in a medium containing protease. After gel

separation, the resulting contents were analysed for the presence of free MTX using HPLC. Fig. 4 shows a HPLC chromatogram of the hydrolysis products of [Fe-PGNP]-PL-MTX. A similar chromatogram was obtained from the hydrolysis products of PL-MTX. It was found that the hydrolysed components of these formulations does not contain any free drug, or the MTX degradation product N^{10}-mFA. As mentioned earlier, free MTX is not necessary for the cytotoxic action because the degradation products of PL-MTX are active against tumor cells (12,13). Interestingly, three peaks, A, B and C were detected in the hydrolytic products of PL-MTX and [Fe-PGNP]-PL-MTX. A preliminary control experiment confirmed the stability of MTX under these conditions. It is known that the enzymatic hydrolysis of PL-MTX results in the liberation of MTX-oligopeptides (7). Hence it is possible that the peaks A, B and C from the hydrolysis products of PL-MTX and [Fe-PGNP]-PL-MTX are due to similar drug-oligopeptides. Further work is required to confirm this possibility. Nonetheless, appearance of three similar peaks from the hydrolysed components of PL-MTX as well as [Fe-PGNP]-PL-MTX is encouraging. This tends to indicate that the enzymatic lysis of these two formulations occurs in a similar fashion.

The size of Fe-PGNP used for bonding the conjugate ranged between 0.6 to 1.5 μm. In order to offer third-order drug delivery, these particles must cross the vascular endothelium, either by endocytosis or by passage through the endothelial cell gaps, and be taken up by the target cells. Since the process of extravascular transport of magnetic carriers is dependent upon their size (20), it may be necessary to further decrease the size of Fe-PGNP. Previous work carried out in our laboratory has shown that this can be achieved by adjusting the synthetic conditions during the polymerization of monomer (6). Indeed it would also be necessary to increase the entrapment of magnetite in PGNP, so as to allow efficient retention of carrier at the target-site (4,18,20).

In conclusion, the preliminary results of this study indicate the possibility that [Fe-PGNP]-PL-MTX may offer third-order active drug targeting for cancer chemotherapy.

Acknowledgments

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