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TYROSINAMIDE RESIDUES ENHANCE PINOCYTIC CAPTURE OF *N*-(2-HYDROXYPROPYL)METHACRYLAMIDE COPOLYMERS

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_N_-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers have been proposed as a potential lysosomotropic drug delivery system. HPMA copolymers bearing tyrosinamide residues, bound either directly to the polymer backbone or via a glycylglycine spacer, were radiolabelled with [^{125}I]iodide and the effect of tyrosinamide content on their rate of pinocytic uptake by rat visceral yolk sacs cultured in vitro was measured. Incorporation of tyrosinamide enhanced uptake of the copolymer, most markedly at substitutions above 10 mol%. 2,4-Dinitrophenol, an inhibitor of pinocytosis, was used to confirm that tissue association of ^{125}I-radiolabelled copolymer was due to pinocytic uptake. The side-chain -Gly-Gly-Tyr-NH_2 was degraded following the internalization of copolymers containing this spacer and degradation was partially sensitive to the lysosomal thiol-proteinase inhibitor leupeptin. It is postulated that the effect of tyrosinamide residues is to increase the hydrophobicity of poly(HPMA) and thus to increase its capacity for nonspecific adsorptive pinocytosis.

Introduction

Use of macromolecules as targetable drug carriers is receiving increasing attention [1]. Attachment of low molecular weight pharmaceuticals to macromolecules restricts their capture by cells to the mechanism of pinocytosis and gives the potential for directing drugs specifically to cells where their therapeutic effect is required. Polymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) possess many of the attributes necessary for an efficient drug delivery system [2]. Copolymers of HPMA can be synthesized to contain polymer-drug linkages that are stable in plasma [3] but efficiently degraded by lysosomal enzymes [4,5], and incorporation of carbohydrate residues has been used to direct these copolymers to specific target cells in vivo [6]. Synthesis of HPMA copolymers whose polymer chains are connected by biodegradable crosslinks affords a potential solution to any problems arising from retention of this (non-biodegradable) polymer in the body [7,8]. In addition HPMA polymers are known to be inert and non-toxic [9], and those HPMA copolymers so far investigated display little or no immunogenicity [10].

We have already demonstrated [11] that HPMA copolymers containing a small number of oligopeptide side-chains (up to 2 mol%) are captured by fluid-phase pinocytosis by rat visceral yolk sacs cultured in vitro, that is, they are engulfed in the extracellular fluid with little or no adsorption to the pinosome membrane. However, many other substrates enter this tissue by adsorptive pinocytosis, and accumulated data from studies with proteins [12–14] and polymers [15] point to increased hydrophobicity of a pinocytic substrate as a major determinant of increased membrane adsorption

and consequentially increased rate of uptake. In this study we have sought to test this hypothesis by preparing HPMA copolymers with graded content of the same hydrophobic substituent and examining their rate of uptake by the rat yolk sac. Tyrosinamide was the substituent chosen, since it is readily labelled with ^{125}I.

HPMA copolymers were prepared containing increasing amounts of tyrosinamide (Tyr-NH_2) up to a maximum of approx. 20 mol% above which the polymers are insufficiently water soluble. Two series of copolymer were synthesized, the first containing Tyr-NH_2 bound directly to the polymer backbone and the second containing -Gly-Gly-Tyr-NH_2 side-chains. All the copolymers were radioiodinated using the chloramine T method, so as to permit measurement of their uptake by cells. The rate of pinocytic capture of each copolymer was determined, by measuring the rate of accumulation of radioactivity by the yolk sac and, where relevant, the extent of any intracellular degradation of radiolabel-bearing side-chains. The inhibitors 2,4-dinitrophenol and leupeptin were used to confirm that uptake of polymers was by pinocytosis and that degradation of the peptidyl side-chain was by lysosomal enzymes. The effect of substrate concentration on the rate of capture of two of the copolymers was measured.

A preliminary report of these findings has been published [2].

Materials and Methods

Chemicals. ^{125}I-Labelled poly(vinylpyrrolidone) and [^{125}I]iodide (preparations I.M.33P and IMS.30) were from Amersham International, U.K. Tissue culture medium 199 was from GIBCO, Bio-Cult Ltd., Paisley, U.K. 2,4,-Dinitrophenol was from Sigma (London) Chemical Co., Poole, Dorset and leupeptin was from the Peptide Institute, Osaka, Japan. *N*-Methacryloyltyrosinamide was prepared by reaction of methacrylic acid *p*-nitrophenylester and tyrosinamide in dimethylformide. The crude product was recrystallized from ethanol (melting point 192–5°C).

Preparation of HPMA copolymers. Copolymers containing Tyr-NH_2 bound directly to the polymer backbone were prepared by radical precipitation copolymerisation of HPMA with *N*-methacryloyltyrosinamide in acetone with 2,2′-azodiisobutyronitrile (AIBN) as initiator (12.5 wt% monomers, 0.6wt% AIBN).

The copolymers containing -Gly-Gly-Tyr-NH_2 side-chains were prepared using methods previously described [16]. First polymeric precursors were synthesized by the copolymerisation of HPMA with different amounts the *p*-nitrophenyl ester of *N*-methacryloylglycylglycine (0.9–20.0 mol %). The precursor was then aminolysed with Tyr-NH_2, Remaining *p*-nitrophenyl ester-containing side-chains, if any, were subsequently aminolysed with 1-aminpropan-2-ol. In both cases the content of Tyr-NH_2 was estimated spectrophotometrically ($\epsilon = 7.67 \cdot 10^3$ in ethanol). The mean molecular weight and polydispersity of the samples was measured using gel permeation chromatography (Sepharose 4B/6B) and the chemical characteristics of the preparations used are shown in Table 1.

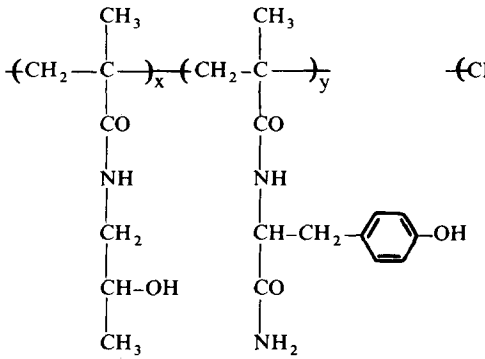
All the HPMA copolymer preparations were radiolabelled using the chloramine T method as previously described [11] and excess [^{125}I]iodide removed by dialysis against 1% NaCl. The specific activity of the preparations was 17–46 μCi/mg polymer.

Estimation of pinocytic uptake of ^{125}I-labelled HPMA copolymers by rat yolk sacs. The tissue accumulation of ^{125}I-labelled HPMA copolymers was measured as previously described for quantifying the uptake of ^{125}I-radiolabelled poly(vinylpyrrolidone) [17]. Yolk sacs were incubated singly in flasks containing 10 ml of medium TC199 without serum [18] and the radiolabelled substrate (1–10 μg/ml) added at the start of an experiment. In experiments where 2,4-dinitrophenol (50 μg/ml), a known inhibitor of pinocytic uptake [19], or leupeptin (40 μg/ml), an inhibitor of lysosomal thiol-proteinases [20], were included, the inhibitors were added simultaneously with the radiolabelled substrate. If tissue accumulation of radioactivity was linear with time, a rate of uptake was calculated. In order to permit direct comparison of experiments that used different concentrations and specific radioactivities of a substrate, rates of uptake were expressed as clearances, in terms of μl of culture medium whose contained substrate was captured per mg yolk sac protein per hour [17].

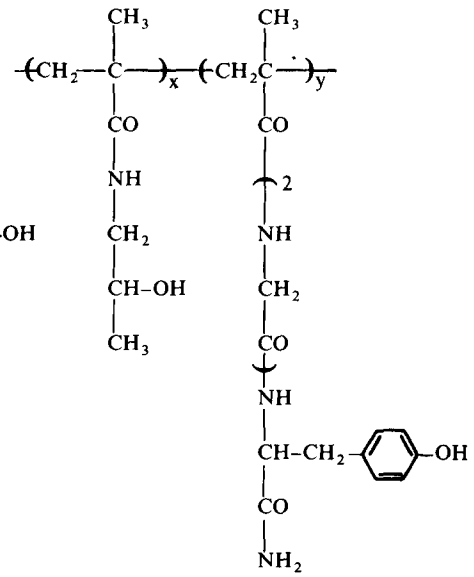
It was known form previous work [11] that,

TABLE I

CHARACTERISTICS OF HPMA COPOLYMERS



Copolymers 1–4



Copolymers 5–12

Code No.	Side-chain composition	Side-chain content (mol%)	M_r	M_r/M_n
1	Tyr-NH_2	0.6	84000	2.9
2	Tyr-NH_2	7.3	46000	2.3
3	Tyr-NH_2	11.5	N.D. [a]	N.D. [a]
4	Tyr-NH_2	15.4	N.D. [a]	N.D. [a]
5	Gly-Gly-Tyr-NH_2	0.9	42000	1.7
6	Gly-Gly-Tyr-NH_2	3.7	24000	1.4
7	Gly-Gly-Tyr-NH_2	9.0	16000	1.4
8	Gly-Gly-Tyr-NH_2	10.9	16000	1.3
9	Gly-Gly-Tyr-NH_2	12.1	13000	1.3
10	Gly-Gly-Tyr-NH_2	13.1	13000	1.3
11	Gly-Gly-Tyr-NH_2	15.4	12000	1.3
12	Gly-Gly-Tyr-NH_2	18.9	10000	1.3

[a] N.D., these values were not determined due to the poor water solubility of these samples.

following pinocytic uptake of HPMA copolymers bearing the oligopeptide side-chain -Gly-Gly-Tyr-*p*-nitroanilide, intralysosomal degradation led to the release of [^{125}I]iodotyrosine back into the incubation medium. To assess whether there was any similar degradation of copolymers containing either of the side-chains -Tyr-NH_2 or -Gly-Gly-Tyr-NH_2, samples of culture medium taken following incubation of yolk sacs with copolymers 4, 10, 11 and 12 were subjected to Sephadex G-15 chromatography. Quantitation of degradation products detected was carried out to give a value representing the volume of culture medium (μl) whose contained substrated had been degraded per mg yolk sac protein [11]. Using the same technique the uptake and degradation of copolymers 4 and 12 in the presence of 2,4-dinitrophenol (50 μg/ml) and leupeptin (40 μg/ml) was evaluated.

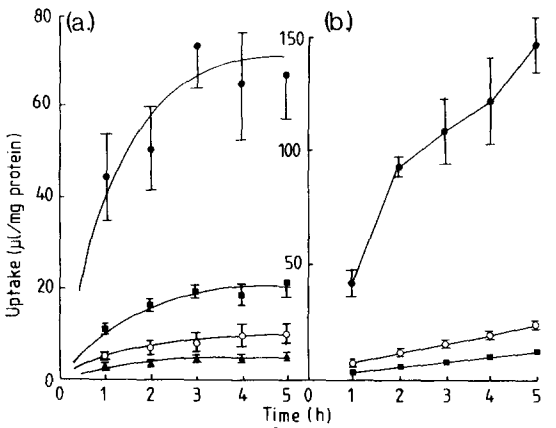


Fig. 1. Accumulation of radioactivity by yolk sacs incubated with ^{125}I-labelled HPMA copolymers. Uptake of Gly-Gly-Tyr-NH_2 side-chain-containing copolymers is shown in: (a) copolymer 5 (▲———▲), copolymer 7 (○———○), copolymer 11 (■———■), copolymer 12 (●———●); and the uptake of Tyr-NH_2-containing copolymers is shown in: (b) copolymer 1 (■———■), copolymer 2 (○———○), copolymer 4 (●———●). Each point represents the mean (±S.E.) of six experimental values.

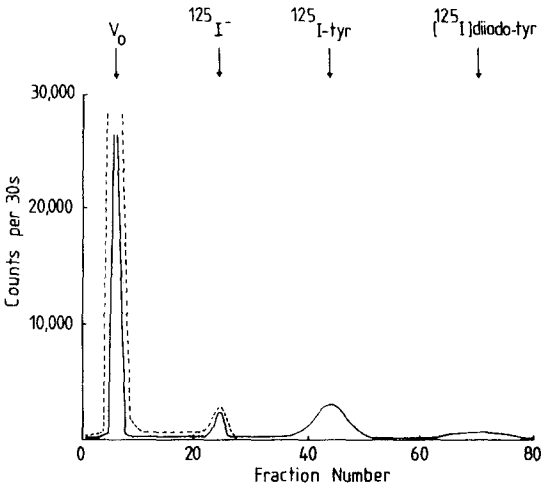


Fig. 2. Evaluation of ^{125}I-labelled HPMA copolymer degradation. Ten yolk sacs were incubated together in 10 ml of medium 199 (for 5h at 37°C) with either ^{125}I-labelled copolymer 4 or 12. After the incubation 1-ml aliquots of culture medium were subjected to Sephadex G-15 column chromatography (column height 28 cm, diameter 1.7 cm) and the resultant fractions (1 ml) were assayed for radioactivity. The elution profiles obtained for copolymers 4 (- - - - -) and 12 (———) are shown. The column was calibrated with blue dextran (V_0), [^{125}I]iodide, 3-[^{125}I]iodo-L-tyrosine and 3,5-[^{125}I]diiodo-L-tyrosine.

Results

Increasing the Tyr-NH_2 content of HPMA copolymers produced an increase in their rate of accumulation by yolk sacs whose extent was related to the degree of substitution with Tyr-NH_2 (Fig. 1). Those copolymers containing Tyr-NH_2 residues bound directly to the polymer backbone

TABLE II

QUANTITATION OF THE DEGRADATION OF COPOLYMERS 4, 10, 11 AND 12. EFFECT OF 2,4-DINITROPHENOL AND LEUPEPTIN

The incubations and subsequent gel permeation chromatography were carried out as described in the Methods section. The results shown are the average of two separate experiments.

Copolymer code no.	Addition to the incubation medium	Tissue accumulation (µl/mg protein)	Degradation (µl/mg protein)	Total uptake (µl/mg protein)	% Degraded
4	none	87.75	0	87.75	0
4	leupeptin (40 µg/ml)	100.16	0	100.16	0
4	2,4-dinitrophenol (50 µg/ml)	5.56	0	5.56	0
10	none	13.06	33.55	46.61	72
11	none	15.20	31.22	46.42	67
12	none	40.73	66.92	107.65	62
12	leupeptin (40 µg/ml)	100.74	19.17	119.91	16
12	2,4-dinitrophenol (50 µg/ml)	4.50	1.27	5.77	22

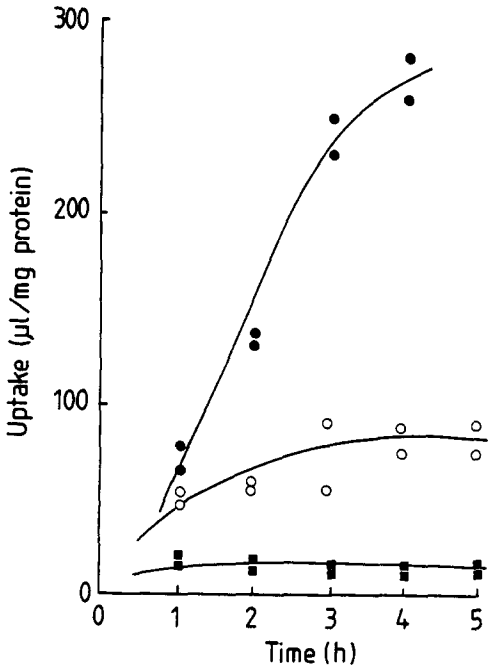


Fig. 3. Typical experiments showing the yolk sac accumulation of ^{125}I labelled copolymer 12 incubated alone (○——○) and in the presence of 2,4-dinitrophenol (■——■) or leupeptin (●——●). Each point represents data obtained from a single yolk sac.

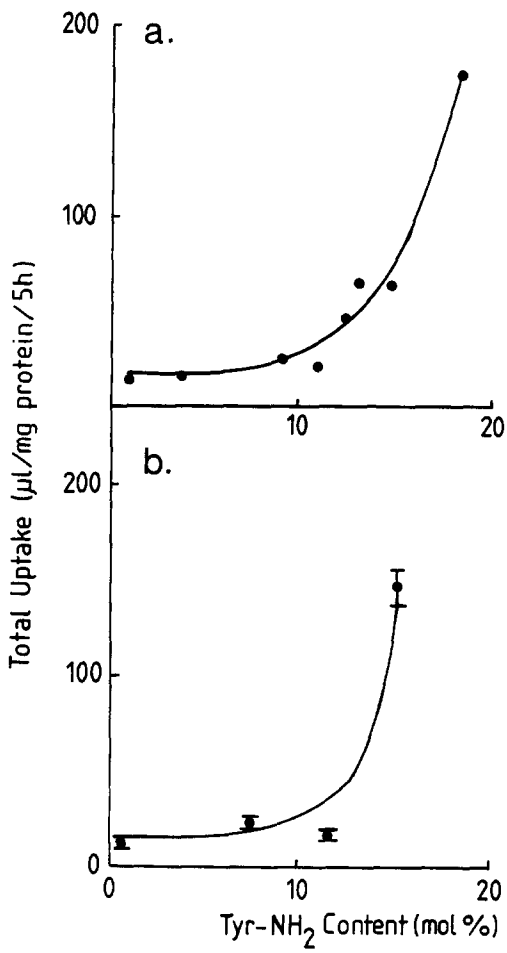


Fig. 4. The relationship between the uptake of ^{125}I-labelled copolymers bearing (a) Gly-Gly-Tyr-NH_2 side chains and (b) Tyr-NH_2 bound directly to the polymer backbone, and the Tyr-NH_2 content of the polymers. Data are expressed as described in the text.

showed linear accumulation of radioactivity over the 5h incubation period, whereas those bearing -Gly-Gly-Tyr-NH_2 side-chains showed a non-linear pattern of accumulation. Chromatography of culture media from incubations of copolymers 4 and 12 with yolk sacs showed (Fig. 2) that copolymer 12 was degraded to yield two low molecular weight degradation products that co-eluted with mono- and di-iodotyrosine, while, in contrast no degradation products were detected from copolymer 4.

The tissue accumulation and, using Sephadex G-15 chromatography, the degradation of copolymers 4, 10, 11 and 12, were quantified under standard incubation conditions and, for copolymers 4 and 12, in the presence of 2,4-dinitrophenol and leupeptin. Table II shows the results obtained and it can be seen that with all the copolymers bearing -Gly-Gly-Tyr-NH_2 side-chains approx. 60–70% of the total radioactivity captured during a 5 h incubation is released back into the culture medium in the form of low molecular weight degradation products. Over a 5 h incubation period 2,4-dinitrophenol effectively inhibited the uptake of copolymers 4 and 12, while leupeptin partially inhibited the degradation of copolymer 12. Leupeptin however had no effect on the rate of pinocytic uptake of copolymers 4 or 12. The effects of dinitrophenol and leupeptin on the uptake and degradation of copolymer 12 were confirmed by measuring the tissue accumulation of this copolymer with time (Fig. 3).

The pinocytic uptake of all the copolymers listed in Table 1 was estimated after a 5 h incubation period. For polymers bearing -Gly-Gly-Tyr-NH_2 side-chains, the total uptake was calculated by

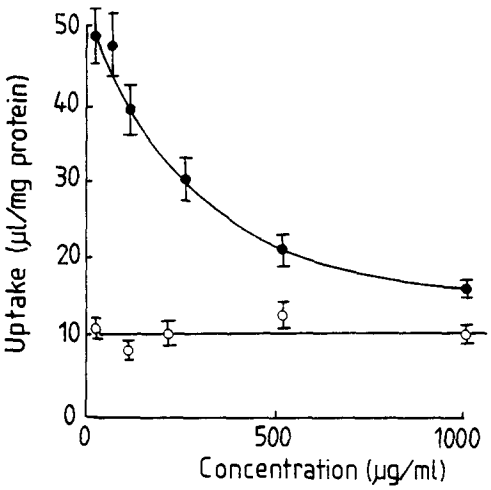


Fig. 5. Effect of copolymer concentration on the uptake of ^{125}I-labelled copolymers 2 (○——○) and 4 (●——●) by yolk sacs. At each substrate concentration ten yolk sacs were incubated together in 10 ml of medium 199 and subsequently assayed separately for the accumulation of radioactivity. The uptake values shown are the mean ± S.D. Copolymer 2 was incubated for 5 h at 37°C, and copolymer 4 for 1 h at 37°C before measurement of yolk sac uptake.

adding to the measured tissue accumulation, either a figure representing the degradation component calculated from the known rates of degradation of copolymers 10, 11 and 12 (Table II) or, in the case of the other copolymers, a value for degradation estimated on the assumption that 67.5% of the radioactivity captured by the yolk sacs was released back into the medium after a 5 h incubation in the form of low molecular weight degradation products. The correlation between total uptake after a 5 h incubation period (µl/mg protein) and the Tyr-NH_2 content of HPMA copolymers is shown in Fig. 4 for both types of copolymer tested.

In all the above experiments a single concentration of copolymer (between 1 and 10 µg/ml) in the incubation medium was used. The effect of substrate concentration on the uptake of two Tyr-NH_2 copolymers was investigated and the results obtained are shown in Fig. 5. The HPMA copolymer bearing the highest amount of Tyr-NH_2 (15.4 mol%) clearly displays a mode of capture that is concentration-dependent. In contrast the other (with a Tyr-NH_2 content of 7.3 mol% appears to be captured at a rate independent of substrate concentration, over the range examined. Lineweaver-Burk analysis of the pinocytic uptake data obtained for copolymer 4 showed K_m and V_{max} values of 489 µg/ml and 24.8 µg/mg protein per h respectively.

Discussion

The results reported support an earlier observation [16] that a water-soluble synthetic polymer substituted with hydrophobic aromatic residues is pinocytosed more rapidly than the parent molecule by rat visceral yolk sacs cultured in vitro. Synthesis of a series of HPMA copolymers bearing different amounts of Tyr-NH_2 has enabled us to probe the relationship between the degree of substitution of the copolymer and its rate of pinocytic capture by the yolk sac.

It was found that the rate of accumulation of HPMA copolymers bearing Tyr-NH_2 directly linked to the polymer backbone increased with increasing Tyr-NH_2 content (Fig. 1). These copolymers were not degraded by the tissue (Fig. 2) and hence their rate of accumulation by the yolk sac with time gives a true measure of their rate of pinocytic capture. Copolymer 4, the copolymer containing the highest amount of Tyr-NH_2 residue was captured at a rate of 24.37 ± 3.65 µl/mg protein/h (mean ± S.E.), an uptake which is some ten times faster than that previously reported for fluid-phase pinocytosis in this tissue [17].

In contrast the Gly-Gly-Tyr-NH_2 side-chains were subject to intralysosomal digestion. Over a 5 h incubation period, some 67% of the radioactivity internalized in association with these copolymers was released back into the incubation medium in the form of low molecular weight degradation products (Table II) and the extent of degradation observed did not appear to vary significantly with the degree of side-chain substitution in the copolymer. This observation permits estimation of the true rates of pinocytic uptake of HPMA copolymers bearing -Gly-Gly-Tyr-NH_2 side-chains. The relationship between Tyr-NH_2 content and rate of uptake was similar to that for copolymers with directly-linked Tyr-NH_2 residues (Fig. 4).

The increased rate of pinocytosis caused by the presence of Tyr-NH_2 residues could theoretically be attributable to one of two phenomena. Either these copolymers are able to stimulate pinocytosis

in this tissue, i.e. to increase the rate of fluid internalization, or they are captured by adsorptive pinocytosis. The first suggestion was eliminated by demonstrating that the Endocytic Index of ^{125}I-labelled poly(vinylpyrrolidone), a marker of fluid-phase pinocytosis in the yolk sac [16], was not altered by addition of copolymers 4 or 12 (100 μg/ml) indicating that they do not stimulate the pinocytic process. Endocytic Indices of 1.59 and 1.57 μl/mg protein per h were measured in the presence of copolymers 4 and 12, respectively, the rate of ^{125}I-labelled poly(vinylpyrrolidone) uptake measured in the absence of copolymer being 1.75 μl/mg protein per h. It is clear, therefore, that the relationship between an increased degree of Tyr-NH_2 incorporation and an enhanced rate of capture (Fig. 4) is attributable to the greater affinities of the more highly substituted copolymers for the yolk sac surface. A markedly enhanced pinocytic uptake, by Tyr-NH_2 or Gly-Gly-Tyr-NH_2, was seen only above a degree of substitution of approx. 10 mol%, This may indicate some gross conformational change in the molecule at this point (perhaps the formation of a hydrophobic domain) which in turn provides a greater affinity for the cell surface. It is noteworthy that recent experiments (Duncan, R., et al., unpublished data) have demonstrated that polyaspartamides of comparable molecular weight average bearing increasing amounts of tyramine show a similar increase in their rate of uptake at a substitution level of approx. 10 mol%.

The ability of 2,4-dinitrophenol (50 μg/ml) to inhibit the capture of copolymers 4 and 12 (Table II) confirms that these macromolecules are progressively internalized by pinocytosis and do not simply bind to the external surface of the tissue [21]. The lysosomal thiol-proteinase inhibitor leupeptin did not alter the rate of capture of HPMA copolymers but, as can be seen from Table II and Fig. 3, this substance inhibited the degradation of the -Gly-Gly-Tyr-NH_2 side-chain by approx. 75%. This observation, together with the fact that no low molecular weight degradation products appeared in the presence of 2,4-dinitrophenol, confirms that hydrolysis of the side-chain is indeed an intracellular event and is largely mediated by lysosomal thiol-proteinase activity. The importance of thiol-proteinases in the lysosomal degradation of peptidyl side-chains in HPMA copolymers has been demonstrated elsewhere [5]. To examine the effect of substrate concentration on the rate of capture of the copolymers, it was only practicable to utilize those with directly attached Tyr-NH_2, i.e. those that are not degraded intracellularly. Copolymers 2 and 4 were selected. It can be seen from Fig. 5 that the uptake (expressed as μl/mg yolk-sac protein) of copolymer 2 is independent of substrate concentration, whereas copolymer 4 shows a decrease in uptake with increasing substrate concentration. This indicates that uptake of copolymer 4 displays typical saturation kinetics whereas the uptake of copolymer 2 does not. The result with copolymer 4 is consistent with entry by adsorptive pinocytosis, but the result with copolymer 2 is puzzling. The mean rate ($\pm$S.D.) of pinocytosis of copolymer 2 measured over a 5 h incubation period (at a substrate concentration of approx. 1 μg/ml) was 4.56 ± 0.28 μl/mg protein per h, compared with the mean value obtained for copolymer 1 of 2.23 μl/mg protein per h. As discussed earlier, the greater rate of capture of copolymer 2 must be due to its capture by adsorptive pinocytosis. However, if this is true, increasing the substrate concentration in the culture medium should cause saturation of the membrane receptors or binding sites, and this was not observed over the concentration range examined. The only plausible explanation of these apparently conflicting data, a greater rate of capture without concentration-dependence, is that the dependent phase of uptake takes place at concentrations below those utilized here. Similar substrate concentration-uptake relationships have been described elsewhere in the literature and non-concentration-dependent and concentration-dependent pinocytic capture has been related to nonspecific and specific binding of substrate, respectively [22].

K_m and V_{max} values were calculated for copolymer 4 in μg units, but conversion to molar terms is not possible, firstly because the preparation is polydisperse and secondly because, owing to its low solubility, estimation of an accurate mean molecular weight is not possible. This precludes direct comparison with the binding constant reported for other substrates of pinocytosis in the yolk sac system.

We have shown that increasing Tyr-NH_2 substitution of HPMA copolymers, whether it be directly attached to the polymer backbone or via a glycylglycine spacer, increases the rate of pinocytic capture of the copolymer. This observation has implications for the proposed use of HPMA copolymers and other synthetic polymers as drug carriers. The hydrophobic interaction with the yolk sac surface which is promoted here by Tyr-NH_2 is likely to be generally applicable to a wide variety of other cell types. A drug carrier loaded with high concentrations of a hydrophobic drug may interact universally with cells even if it bears residues capable of selective targeting to a specified cell type. The relationship between the amount of Tyr-NH_2 in the HPMA copolymer and its rate of pinocytosis shows that, whereas uptake does increase progressively with Tyr-NH_2 levels up to 10 mol%, the most dramatic increase in uptake occurs at higher substitutions. These data obviously recommend, as a general principal, drug loadings of less than 10 mol% in order to minimise the non-specific interaction of HPMA drug carriers bearing hydrophobic compounds.

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