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SYNTHETIC POLYMERS AS TARGETABLE CARRIERS FOR DRUGS

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Abstract - The cellular phenomena of pinocytosis and lysosomal digestion are described, with particular emphasis on the handling of soluble macromolecules by living cells. A drug delivery system based on poly(hydroxypropylmethacrylamide) conjugated to drug analogues by oligopeptide sequences has been evaluated. The conjugates, which are stable in the bloodstream, are captured by pinocytosis and taken to the lysosomal compartment of cells. There the oligopeptide sequences are hydrolysed by the lysosomal enzymes and the drug analogues released. Targeting has been demonstrated by incorporation into the polymer of side-chains bearing moieties that lead to non-specific or cell-specific adsorptive pinocytosis.

INTRODUCTION

Conventional chemotherapy is inefficient in two ways. First, drug loss by excretion and metabolism increases the dose required and also produces suboptimal pharmacokinetic behaviour. Secondly, the ubiquitous body distribution of most drugs frequently leads to unwanted side-effects as well, of course, as being wasteful. It is therefore not surprising that a major goal of pharmaceutical research is the organ- and cell-specific targeting of drugs.

Macromolecules, both synthetic and naturally occurring, have already won a place in these new approaches to drug delivery. Insoluble polymers are being used as matrices in which drug is entrapped for controlled release within the body. By this means, advances have already been made, and more are likely, in improving the pharmacokinetic behaviour of many medicaments.

A more ambitious aim is the use of macromolecules to deliver drugs to pre-selected sites in the body. The macromolecule used would require features enabling it to recognise and enter particular cell-types, and ideally the drug would not be released in active form until the conjugate was inside the target cell. The work to be reported here demonstrates that this approach to chemotherapy is a realistic one.

HANDLING OF MACROMOLECULES BY LIVING CELLS

Macromolecules cannot, in general, cross biological membranes. In consequence they remain much longer in the compartment of the body (or of the individual cell) into which they are introduced. Thus soluble macromolecules injected into the bloodstream remain there for long periods, whereas small molecules pass rapidly into cells throughout the body, and are also lost into the urine following passage through the kidney glomerulus.

Although macromolecules cannot cross membranes, there is a mechanism whereby they can enter cells. This mechanism, known as endocytosis, involves the pinching-off of a fragment of a cell's limiting membrane to form a small vesicle within the cytoplasm. Two sub-sets of endocytosis are recognized : pinocytosis - the formation of small vesicles that in general can contain only dissolved materials, and phagocytosis - the formation of rather larger vesicles that contain micro-particulate material. There are important differences between pinocytosis and phagocytosis. First, pinocytosis appears to be a constant activity of cells, whereas phagocytosis occurs sporadically in response to the arrival at the cell's surface of a suitable particle for ingestion. Secondly, pinocytosis is a feature of nearly all cell-types, while phagocytosis is the preserve of only a few, those that are 'professionally' engaged in removing offending particles such as micro-organisms from the extracellular fluids of the body.

Although pinocytosis is a constitutive activity of cells, i.e. it is not substrate-triggered, it is nevertheless capable of very considerable substrate-specificity. Any soluble substance present in the ambient fluid will be captured by pinocytosis, but a substance that has an affinity for the plasma membrane will be selectively internalized. Substances captured thus by 'adsorptive pinocytosis' may be very efficiently cleared from the fluid around a cell and even from a moving stream of fluid passing over a cell. A good deal has been learnt in recent years about the features required by a macromolecule in order to be selectively captured by adsorptive pinocytosis. A particularly noteworthy finding is that cell-types differ in their affinities for particular moieties; this property of cells has obvious potential for exploitation in drug targeting.

The usual fate of a pinocytic or phagocytic vesicle is fusion with one or more lysosomes. Lysosomes are resident intracellular vacuoles that contain a high concentration of hydrolytic enzymes which between them are capable of degrading the many amide (peptide), ester and glycosidic linkages found in biopolymers. Some fifty distinct enzymes have been identified, each with its own substrate specificity. Fusion with lysosomes brings the contents of the pinocytic or phagocytic vesicle into contact with the lysosomal enzymes. Whether digestion of the contents occurs within this composite vacuole (known as a digestive vacuole, or a secondary lysosome) depends on the susceptibility of the macromolecules captured to the enzymes of the lysosomes. Substances that are not degraded remain within the lysosomes for long periods, often for the life of the cell. Excretion of indigestible macromolecular or particulate material from the lysosomes is not a feature of most cell-types.

The membrane surrounding the lysosome is impermeable to macromolecules. It does however permit the passage of the monomers of which biopolymers are composed, viz. monosaccharides, amino acids, nucleosides. Many drugs, particularly if their chemical structure contains significant hydrophobic regions, should also be able to cross from lysosomal interior to cytoplasm, and thence to other structures such as the nucleus and the ribosomes.

DESIGN OF A TARGETABLE DRUG-POLYMER CONJUGATE

These basic facts of cell biology must be the starting point for any project whose objective is the design of a targetable polymeric carrier for intracellular drug delivery.

There are several encouraging features. A drug-polymer conjugate injected into the bloodstream would not pass into the glomerular filtrate. Nor would it enter cells indiscriminately by crossing cell membranes. The phenomenon of cell-type-specific adsorptive pinocytosis holds promise for targeting macromolecules to selected sites in the body. And the existence and properties of the lysosomal enzymes engender the concept of a drug-polymer linkage that is stable in transit to the target cell, but broken on arrival inside the lysosomes of that cell.

There are also limitations. A drug to be conjugated will need to be a small enough molecule to pass across the lysosome membrane, in order to reach the subcellular site at which its action is desired. It will also need to be resistant to the action of the lysosomal hydrolases, once it has been freed from macromolecular carrier. However the polymer to be used as carrier should ideally be degradable by the lysosomal enzymes, in order to avoid accumulation within the target cells. And finally, parenteral administration would seem to be required : polymers are poorly absorbed from the gut, and drug-polymer linkages might be susceptible to the digestive enzymes.

We now describe experiments in which a soluble synthetic polymer, conjugated to drug analogues and bearing targeting moieties, has been evaluated as a potential drug delivery system. Emphasis will be on cell-specific pinocytic capture and on the handling of the conjugate within the lysosomes.

POLY(HYDROXYPROPYLMETHACRYLAMIDE) - A VERSATILE DRUG-CARRIER

Our experiments have been carried out with methacrylamide-based copolymers of M_w approx. 50 000. A variety of side-chains can be introduced by substitution through the amide linkage of oligopeptides or other molecules that bear a primary amino-group. Remaining monomer units bear 1-aminopropan-2-ol residues. The copolymers are synthesized by radical copolymerization of hydroxypropylmethacrylamide with methacryloyl derivatives of appropriate amines. As will be explained below, it is possible to achieve subsequent side-chain substitutions, to produce a copolymer bearing both drug and targeting moieties.

OLIGOPEPTIDES AS THE DRUG-POLYMER LINK

As explained above, the drug-polymer link must be hydrolysed by the lysosomal enzymes. Using p-nitroaniline as a drug analogue, we have measured its release from hydroxypropylmethacrylamide copolymers bearing a variety of oligopeptidyl p-nitroanilide side-

chains, on incubation with a lysate of rat liver lysosomes.

The copolymers were synthesized by copolymerization (as described above) with the p-nitrophenyl ester of a methacryloyl di- or tri-peptide, followed by reaction of the copolymer with aminoacyl or oligopeptidyl p-nitroanilide.

Lysosomal enzymes released p-nitroaniline from many of the copolymers. Side-chain susceptibility varied greatly with the length and amino acid composition, but satisfactory rates of cleavage were achieved with a number of tri- and tetrapeptide side-chains. We have strong evidence that the thiol-activated peptidases of the lysosomes are the enzymes responsible.

The results are described fully elsewhere (Ref. 1-3) and are also discussed in two recent published lectures (Ref. 4 & 5).

MOLECULAR FEATURES THAT ENHANCE ADSORPTIVE PINOCYTOSIS

Poly(hydroxypropylmethacrylamide) has no intrinsic affinity for cell surfaces and so enters cells by pinocytosis in the bulk fluid phase only. It therefore provides an ideal molecule for determining whether particular side-chain substituents lead to uptake by adsorptive pinocytosis.

We have recently investigated the mode of pinocytic uptake, in an epithelial cell-type, of copolymers bearing either tyrosinamide or glycylglycyltyrosinamide side-chains (Ref. 6). Substitutions of up to 10 mol% had little effect on rate of pinocytic uptake, but further increase, up to 20 mol%, led to rates up to ten times the basal fluid-phase rate. There is evidence that hydrophobic moieties in proteins lead to adsorptive pinocytosis (Ref. 7), and we believe that hydrophobicity is the feature of our tyrosinamide-containing polymers that leads to their enhanced uptake.

Molecular size and cationic character are two other parameters that lead to enhanced pinocytic uptake of a macromolecule. The subject of substrate selection in endocytosis has recently been reviewed (Ref. 8).

Reference was made earlier to cell-specific adsorptive pinocytosis. Several examples are known, but the best characterized is the uptake of desialylated glycoproteins by hepatic parenchymal cells. A receptor on the cell surface recognizes the terminal galactose moeity of the oligosaccharide chains of these modified glycoproteins, resulting in rapid uptake from the ambient medium. The mechanism is so efficient that desialylated glycoprotein molecules are removed from the circulation within minutes of their introduction. In the next Section we describe an experiment in which this recognition system on liver cells has been used to deliver a polymer conjugate of a drug analogue.

INTRACELLULAR DRUG DELIVERY

We now describe two experiments that demonstrate the pinocytosis of a polymeric carrier and the subsequent release of the conjugated drug analogue within the lysosomes.

In the first experiment (Ref. 9) four copolymers bearing oligopeptidyl p-nitroanilide side-chains were incubated with an organ culture of the rat yolk sac, a tissue active in pinocytosis and much used in quantitative studies of the phenomenon. The terminal amino acid in each copolymer was tyrosine, permitting their radiolabelling with ^{125}I to yield a penultimate [^{125}I] iodotyrosine, which serves in the experiment as a drug analogue. The four side-chains were of widely differing susceptibility to hydrolysis by lysosomal enzymes, as judged by the experiments in a cell-free system.

When the copolymer bearing the side-chain -Gly-β-Ala-[^{125}I]Tyr-nitroanilide was added to the incubation medium, radioactivity accumulated steadily in the tissue, but no low molecular weight radioactivity appeared in the medium. This is consistent with pinocytic uptake and accumulation with the lysosomes, owing to the non-susceptibility of the side-chain to the lysosomal enzymes. (The β-alanine residue no doubt is unfavourable to attack by these enzymes). When copolymers bearing susceptible side-chains (e.g. Gly-Gly-[^{125}I]Tyr-nitroanilide) were used in the same experiments, the results were different. Radioactivity in low molecular weight form, identified as [^{125}I]iodotyrosine was progressively released from the tissue into the incubation medium. Copolymer was being pinocytosed, followed by side-chain digestion, and release of [^{125}I]iodotyrosine from the lysosome into the cytoplasm. That this interpretation is correct was confirmed by adding to the medium an inhibitor of the thiol-dependent lysosomal peptidases. Release of [^{125}I]iodotyrosine was halted or diminished, and radioactivity instead accumulated within the tissue.

This experiment clearly demonstrates the possibility of drug delivery to cells by the pinocytic uptake and intralysosomal processing of a covalently bound polymeric conjugate.

The second experiment (Ref.10) demonstrates both drug delivery and targeting. A copolymer with two distinct types of oligopeptide side-chain was prepared by consecutive aminolysis of a precursor bearing glycylglycine p-nitrophenyl ester side-chains. First tyrosinamide (0.5 mol %) was bound, to give a [^{125}I] labelable drug analogue attached to the polymer backbone by a diglycyl moiety which would be expected to be cleavable by lysosomal enzymes. Next D-galactosamine (2 mol%) was bound, to mimic the recognition moiety of desialylated glycoproteins that interacts with the hepatocyte plasma membrane.

When this bifunctional copolymer was injected intravenously into the rat, removal from the bloodstream was rapid, and significantly faster than that of equivalent copolymers bearing D-glucosamine or D-mannosamine rather than D-galactosamine residues. Examination of body tissues one hour after injection showed 70% of the radioactivity administered to be present in the liver. Around 10% of the control polymers was found in the liver. Thus galactosamine residues attached to a wholly unnatural polymer are recognized by the liver cell's receptor for galactose-terminating oligosaccharide chains of glycoproteins, leading to selective pinocytosis by hepatocytes.

After five hours radioactivity had largely disappeared from the liver, and was found in the urine. Evidently the diglycyl tyrosinamide side-chains had been cleaved by the lysosomal enzymes, releasing the labelled drug analogue.

CONCLUSION

In this brief account of recent work in our two laboratories, much has been omitted. The stability of our conjugates in plasma (Ref.11), their pleasingly weak immunogenicity (Ref.12) and the potential problem of their non-biodegradability (Ref.13) have all been investigated. But we have focused here on our clear demonstration that synthetic polymers can be targeted and can deliver and release bound substances at an intracellular site. Thus there is a sound basis for further work.

The next phase of the project must be to progress from drug analogues to drugs and from model cell systems to the real targets of chemotherapy. The first objective is within reach, although not without problems. We already have (unpublished) data showing that a polymer-bound oligopeptide from which lysosomal enzymes readily release p-nitroaniline is not susceptible when conjugated to another compound. Each drug to be bound will require work to identify a suitable 'spacer' to link it to the polymer backbone. For the second objective we await further data on the cell-specific determinants for receptor-mediated pinocytosis. Happily this is an area of cell biology in which activity and expectations are high.

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REFERENCES

1. R. Duncan, H.C. Cable, J.B. Lloyd, P. Rejmanová and J. Kopeček, Bioscience Rep. 2, 1041-1046 (1982).
2. R. Duncan, H.C. Cable, J.B. Lloyd, P. Rejmanová and J. Kopeček, Makromol. Chem. 184, 1997-2008 (1983).
3. P. Rejmanová, J. Pohl, M. Baudyš, V. Kostka and J. Kopeček, Makromol. Chem. 184, 2009-2020 (1983).
4. J.B. Lloyd, R. Duncan, J. Kopeček and P. Rejmanová, In Receptor-Mediated Targeting of Drugs (G. Gregoriadis and J. Senior, eds)., Pergamon, New York, 1984.
5. J. Kopeček, P. Rejmanová, R. Duncan and J.B. Lloyd, Ann. N.Y. Acad. Sci., in press.
6. R. Duncan, H.C. Cable, P. Rejmanová, J. Kopeček and J.B. Lloyd, Biochim. Biophys. Acta, in press.
7. J.B. Lloyd and K.E. Williams, Biochem. Soc. Trans. 12, 527-528 (1984).
8. J.B. Lloyd, M.K. Pratten, R. Duncan, T. Kooistra and S.A. Cartlidge, Biochem. Soc. Trans., in press.
9. R. Duncan, P. Rejmanová, J. Kopeček and J.B. Lloyd, Biochim. Biophys. Acta. 678, 143-150 (1981)
10. R. Duncan, J. Kopeček, P. Rejmanová and J.B. Lloyd, Biochim. Biophys. Acta 755, 518-521 (1983).
11. P. Rejmanová, J. Kopeček, R. Duncan and J.B. Lloyd, submitted for publication.
12. B. Říhová, K. Ulbrich, J. Kopeček and P. Mančal, Folia Microbiol. 28, 217-227 (1983).
13. S.A. Cartlidge, R. Duncan, J.B. Lloyd, P. Rejmanová and J. Kopeček, Proc. Internat. Conf. Biomedical Polymers, Durham, 289-292 (1982).