A biospecific polymeric carrier for polypeptide drugs

L. I. Valuev, G. A. Sytov, I. L. Valuev, M. V. Ul´yanova, L.V. Vanchugova, N. A. Platé*

A. V. Topchiev Institute of Petrochemical Synthesis, Russian Academy of Sciences
29 Leninsky prosp., 119991, Moscow, Russian Federation.
Fax: +7 (095) 230 2224. E-mail: valuev@ips.ac.ru

Polymeric systems for targeting polypeptide medicinal drugs onto the wall of the small intestine after oral administration were synthesized. These systems are composed of particles of ovomucoid-modified polyacrylamide hydrogel with insulin physically immobilized in its bulk. *In vitro* experiments have demonstrated the enhanced stability of the immobilized polypeptide against proteolysis and the affinity of polymeric particles to lectin-containing surfaces. The efficiency of the systems obtained was demonstrated in experiments with animals and diabetes patients. The effrct of these systems upon oral administration was qualitatevely similar to that of subcutaneously administered native insulin.

Key worlds: insulin, polymeric derivatives, hydrogels, inhibitors of proteolytic enzymes.

A difficult and basic challenge of the modern chemistry of physiologically active substances is development of the methods for regulation of protein and polypeptide stability against the action of proteolytic enzymes. This problem is especially topical as applied to the design of certain drugs. It is known that vital substances of the polypeptide nature (enzymes, inhibitors, hormones, and so on) are endogeneous rather than are supplied with food. The latter is impossible as proteolytic enzymes that are involved in digestion hydrolyze proteins to amino acids. Therefore, to eliminate protein deficiency, one has to administer the proteins into the organism as injections bypassing the digestive system.

The pancreatic hormone insulin, whose lack or deficiency causes diabetes, is an example of such a protein.

Normally, insulin reaches the liver from the pancreas through the blood vessels connected to the portal hepatic vein. The digestion products are transported to the liver through the same vein. Since the key function of insulin is to control the subsequent transformations of the digestion products, under natural conditions, insulin enters the liver simultaneously with the products. The liver, in turn, controls the amount of insulin that reaches other organs and tissues.[1] No control of this sort is possible for insulin injections (which is yet the only way of diabetes treatment); this causes aftereffects of the pancreatic diabetes, such as cardiovascular diseases, brain function disorder, and so on. Therefore, oral administration of insulin, which mimics the natural secretion tract of this hormone would be the best way from the physiological standpoint.

Advantages of this method of insulin administration are so obvious that attempts at developing insulin drugs able, in the active state, to penetrate the blood through the mucous membrane of the intestine have never stopped since the discovery of the therapeutic action of this hormone in 1922.[2] These attempts include modification of the insulin molecule (replacement of the C-terminal amino acid residue, reactions with natural or synthetic polymers);[3,4] joint use of the hormone and proteinase inhibitors or substances that increase the permeability of small intestine walls (detergents, fatty acid salts, crown ethers);[5,6] coating of insulin preparations with a polymeric film that survives in the stomach but dissolves in the small intestine;[7,8] and the introduction of insulin into liposomes or nanocapsules.[9—12] Although in many cases the hormone stability against the action of proteolytic enzymes increased, no oral insulin forms suitable for clinical use have been developed so far. It has been noted that with conventional approaches, a real effect is attained upon introduction of enormous insulin doses (higher than the doses of injection insulin by a factor of hundreds).[13] Moreover, there is often no correlation between the amount of insulin introduced and the decrease in the blood glucose level.[14] In addition, many studies point to the "phenomenal" difference in the response of different species of animals or even individuals of the same species to the introduced insulin.[15]

The experience we accumulated in the development and use of biospecific sorbents[16—18] allowed us to propose and implement an original idea, namely, to employ biospecific interactions for targeted transport of insulin-containing polymeric hydrogel particles to the small intestine mucous membrane. Studies along this line were initiated at the Topchiev Institute of Petrochemical Synthesis in the early 1990s.[19,20] This paper summarizes the most prominent results obtained during the last decade,

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which culminated in the development of an insulin drug for oral administration called Ransulin.[21—23]

The key problems involved in the oral administration of any protein is protection of the protein from enzymatic hydrolysis and targeted transport onto the mucous membrane of the small intestine where the drug is absorbed into the blood. We proposed ovomucoid-modified polyacrylamide hydrogel laden with physically immobilized insulin as the system capable of solving both problems. Ovomucoid is a natural glycoprotein with a molecular mass of about 31 kDa, a highly efficient inhibitor of a number of proteolytic enzymes.[24] Subsequently, compositions based on polyacrylic acid,[25,26] chitosan and its derivatives,[27,28] 2-hydroxypropyl(methyl)cellulose and other polysaccharides[29] have been widely used as the polymeric carriers of this kind, which were called mucoadhesive polymers.

The wall of the small intestine is known to lectins, *i.e.*, proteins that form complexes with carbohydrates.[29] Therefore, a carbohydrate-containing biopolymer is an appropriate ligand for binding a polymeric hydrogel particle to the intestine wall, while an enzyme inhibitor chemically bound to the polymer can protect insulin, physically immobilized in the hydrogel, from the action of enzymes. The use of ovomucoid that contains polypeptide and oligosaccharide fragments as both a trypsin inhibitor and a modifier of the polymeric hydrogel provides simultaneous solution to both problems.

The modified hydrogel was synthesized by radical copolymerization of acrylamide with *N*,*N*′-methylenebisacrylamide and an unsaturated ovomucoid derivative that has been obtained by acylation of the free amino groups of the inhibitor with acryloyl chloride.[16] The insulin-containing preparations were obtained by incubation of freeze-dried modified hydrogel in an insulin solution.[20]

A study of the interaction of ovomucoid with lectin, namely, concanavalin A (Con A), has shown[23] that ovomucoid as a glycoprotein forms complexes with both native and polymeric hydrogel-immobilized Con A with binding constants of about 10^3 L mol^{-1}. Simultaneously, the polypeptide portion of the ovomucoid molecule interacts with serine proteinases (trypsin and α-chymotrypsin) with binding constants of about 10^8 L mol^{-1} and thus inhibits the activity of these enzymes.[24] The antitryptic activity of ovomucoid within its complex with Con A amounts to 95—97% of the activity of the original ovomucoid.

For comparison, a polyacrylamide hydrogel modified with ovomucoid whose carbohydrate component had been removed by treatment with α-amylase was also used as the carrier. This deglycosylated ovomucoid derivative inhibited proteinases but did not interact with lectins.

The results of investigation into the stability of various insulin preparations to the action of trypsin are shown in Fig. 1. The physical immobilization of insulin in the bulk of the modified hydrogel actually results in its substantially higher stability against the enzyme. The degree of the increase in the insulin stability does not depend on the presence of carbohydrates in the inhibitor molecule.

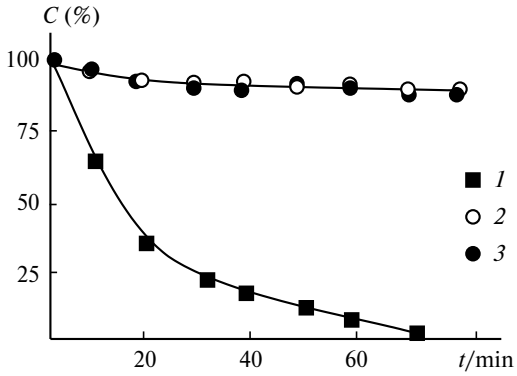

Fig. 1. Concentration of insulin (*C*) *vs.* time of its incubation with trypsin (t = 30 °C, pH 8.0, insulin : trypsin molar ratio 200 : 1): (*1*) native insulin, (*2*) insulin in the ovomucoid-modified hydrogel; (*3*) insulin in the hydrogel modified with deglycosylated ovomucoid (the concentration was calculated in percent of the initial).

In the digestive system, the hydrogel has to pass through areas with different pH and since insulin is not chemically bound to the hydrogel, it was necessary to study the kinetics of insulin release from the hydrogel particles into the surrounding solution at different pH values. The results of this study are presented in Table 1.[30]

At pH 2.5 and 8.0 insulin can freely diffuse from the hydrogel, a uniform distribution between the hydrogel and water being attained as soon as after 90 min. A different situation is observed at pH 4.3, in particular, the final concentration of insulin in the hydrogel bulk is substantially higher than its concentration in the surrounding solution. The amount of insulin associated with the hydrogel does not depend on the volume ratio of the hydrogel and the surrounding solution, being determined only by the amount of ovomucoid in the hydrogel.

Most likely, this effect is due to the electrostatic interaction between ovomucoid and insulin, which have different isoelectric points (5.5 for insulin and 3.8 for ovomucoid). When the pH is intermediate between these two points, the molecules of the protein have opposite charges, so that electrostatic forces prevent free diffusion of insulin. The replacement of water by a 0.1% solution of NaCl eliminates the nonuniform distribution of insulin between the hydrogel and the solution, thus confirming the electrostatic nature of the interaction between the proteins.

The diffusion of insulin from the hydrogel particles through the mucous membrane was studied at pH 7.5 (this corresponds to the pH of the small intestine environment) using a model of the intestine, representing a vessel with porous walls filled with 3% polyacrylamide hydrogel containing Con A.[31] The highest rate of protein

Table 1. Kinetics of washing-out of insulin from the ovomucoid-modified hydrogel[a] at various pH values

V_s/mL[b]	pH	Amount of insulin/mg			
		in the hydrogel after			sorbed[c]
		30 min	60 min	90 min	
1	2.5	0.77	0.63	0.51	0
	4.3	0.81	0.73	0.66	0.16
	8.0	0.76	0.62	0.49	0
2	2.5	0.66	0.46	0.34	0
	4.3	0.69	0.55	0.47	0.14
	8.0	0.66	0.44	0.33	0
3	2.5	0.59	0.35	0.24	0
	4.3	0.64	0.48	0.39	0.14
	8.0	0.58	0.33	0.25	0
1[d]	4.3	0.77	0.64	0.5	0
2[d]	4.3	0.68	0.45	0.32	0
3[d]	4.3	0.57	0.32	0.22	0

[a] The hydrogel volume is 1 mL, the ovomucoid content in the hydrogel was 9.8 mg ml^{-1}, the initial concentration of insulin in the gel was 1 mg mL^{-1}.
[b] The volume of the surrounding solution.
[c] The amount of insulin sorbed was determined from the difference between the total amount of insulin in the gel and the amount of insulin calculated assuming its uniform distribution between the solution and the gel.
[d] The surrounding solution contains 0.1% NaCl.

diffusion at the initial instant was found for ovomucoid-modified hydrogel particles (Fig. 2). The rate of insulin diffusion from the hydrogel modified by deglycosylated ovomucoid was much lower. This means that the decisive contribution to the enhancement of insulin penetration through the vessel walls is made by biospecific binding of the carbohydrate moiety of ovomucoid to lectin (the constant for the interaction of Con A with the immobilized ovomucoid is $(2.2\pm0.7)\cdot10^3$ L mol^{-1}), which ensures adhesion of the polymer particle directly to the reactor walls, and, hence, an increased local concentration of insulin.

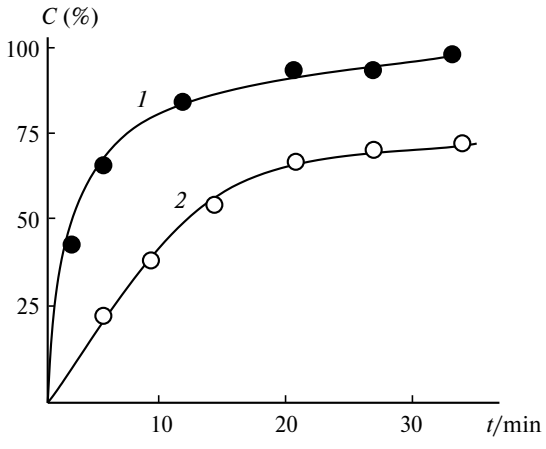

Fig. 2. Diffusion of insulin through the reactor wall made of hydrogel particles modified with ovomucoid (*1*) and deglycosylated ovomucoid (*2*) (*C* is the amount of liberated insulin).

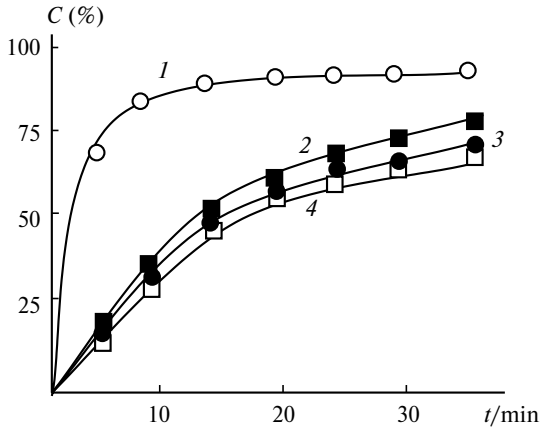

Fig. 3. Diffusion of insulin through reactor wall made of hydrogel particles modified with ovomucoid. Particle diameter/mm: <0.05 (*1*), 0.05—0.2 (*2*), 0.2—1.0 (*3*), 1.0—2.0 mm (*4*).

It is clear that a substantial role of biospecific interactions and their influence on the adhesion of the polymer particle can be manifested only for small particles deeply penetrated into the membrane layer, which ensures the largest number of contacts between the carbohydrate and lectin.

To determine the optimum size of the hydrogel particles for the targeted transport, we studied the fractions with particle diameters of less than 0.05 mm, 0.05—0.2 mm, 0.2—1.0 mm, and 1.0—2.0 mm. The maximum rate of diffusion was found for particles with diameters of less than 0.05 mm (Fig. 3). As will be shown below, these species exhibit the highest activities in experiments with animals (Fig. 4).

The obtained data on the time variation of insulin concentration in the blood of animals on subcutaneous injection of an insulin solution and on oral administration of insulin in the hydrogel[32] are shown in Fig. 5.

It can be seen that after the injection of an insulin solution, its concentration in the blood increases, reaches a maximum after 1.5 h, and then starts to decrease. A nearly the same pattern is observed following the oral administration of insulin within an ovomucoid-modified hydrogel. As expected, oral administration of the drug based on deglycosylated ovomucoid-modified hydrogel does not change the blood insulin concentration, although the protective effect of this ovomucoid derivative is retained (see Fig. 1). This implies that the biospecific lectin—carbohydrate interaction actually plays the crucial role in insulin penetration through the mucous membrane.

Insulin enters the blood in an active state, which results in a decrease in the glucose concentration (see Fig. 5).

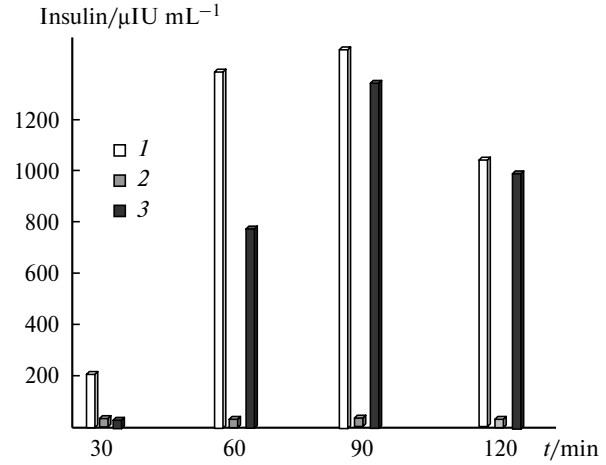

Fig. 4. Blood insulin concentration in white rats after single administration of insulin: (*1*) native insulin, subcutaneously; (*2*) insulin in the hydrogel modified with deglycosylated ovomucoid, orally; (*3*) insulin in the hydrogel modified with ovomucoid, orally. Dose 5 IU kg^{-1} (average for 25 animals); t/min the time after drug administration.

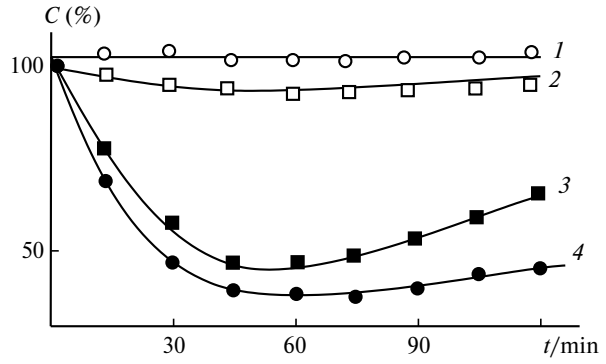

Fig. 5. Glucose concentration (C_g) in blood of rabbits after administration of 5 IU kg^{-1} of insulin (the average for 14 animals): (*1*) control (without insulin), (*2*) subcutaneous injection, (*3*, *4*) oral administration in the hydrogel with ovomucoid with a size of hydrogel particles of less than 0.05 mm (*3*) and 0.1—0.15 mm (*4*).

The efficiency of action of the preparation with a particle size below 0.05 mm after oral administration is only slightly inferior to the efficiency of a subcutaneously administered insulin.[19]

Extended trials of the drug in animals (about 700 rabbits, while rats, and mice) showed a reliable decrease in the blood glucose level, by at least 20% for insulin doses of about 5—8 IU kg^{-1} for 88% of animals. It is significant that the hypoglycemic action is detected both in healthy animals and in animals with experimental diabetes.

All these results, together with the lack of embryotoxic, teratogenic, allergenic, and immunotoxic effects of the drug as well as the absence of influence on the reproductive function, enabled testing of the drug in healthy volunteers and in diabetes mellitus patients.*

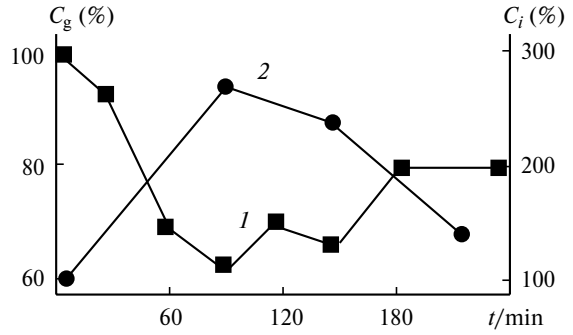

Fig. 6. Variation of the glucose concentration (C_g) (*1*) and insulin (C_i) (*2*) in blood of healthy volunteers after oral administration of 40 IU of the Ransulin preparation.

The tests were carried out in volunteers (20 persons) having fasted for 12 h. The insulin dose was 40 IU. The results are shown in Fig. 6.

It can be seen that oral administration of the drug induces statistically reliable hypoglycemic effect in healthy volunteers. The most pronounced decrease in the blood glucose concentration, on average, 34.9±4.4%, which was attained in 90 min. The reliable increase in the blood insulin concentration after administration of the drug, almost coinciding in time with the lowest blood sugar concentration, confirms objectively the ingress of exogenous insulin to the blood flow.

The group of diabetes patients included 30 persons, 15 with type I diabetes and 15 with type II diabetes, who took injections of insulin.

The amounts of insulin injected in the usual therapy in type I and type II diabetes patients, were naturally different for each of 30 patients, but the average level over the 3 h during which the glucose level was measured amounted from 10 to 30 IU of insulin.

The duration of trials was 3 days for each group of patients. The first day, the glucose concentration was measured in all patients on an empty stomach. The usual doses of injection insulin were administered in patients, and then the blood glucose concentration was measured every 30 min (the patients had breakfast 30 min after the insulin injection). On the second day, instead of insulin injection, the patients took, on an empty stomach, the hydrogel containing 40 IU of insulin, and the third day, they took the hydrogel containing 80 IU of insulin on an empty stomach.

These investigations (Table 2) revealed comparable changes in the blood following glucose level the basic

* The studies complied with the recommendations for Nontherapeutic Biomedical Research Involving Human Subjects adopted at the 18th World Medical Assembly (Helsinki, Finland, 1964).

Table 2. Glucose concentrations in blood of diabetes mellitus patients (% of the init. ±7%) upon administration of native insulin (by injection) and the Ransulin drug (orally)

Preparation	C_g after administration (%)					
	Initial instant	30	60	90	120	150
		min				
	Type I diabetes					
Insulin	100	107	129	149	152	—
Ransulin, 40 IU	100	112	153	183	202	—
Ransulin, 80 IU	100	111	127	155	172	—
	Type II diabetes					
Insulin	100	102	109	142	144	134
Ransulin, 40 IU	100	104	128	153	157	147
Ransulin, 80 IU	100	100	117	137	137	124

injection therapy and oral administration of the insulin-laden hydrogel, on average, in 76% of the type I and type II diabetes patients. For patients with type II diabetes, the oral administration of the drug ensured the control over the blood glucose level similar to that attained by the basic injection therapy in 93% of cases. For most of the patients, a reliable correlation was observed between the glucose concentration and the drug dose.

The system we elaborated is versatile and applicable to any protein.[33] The results of investigations of the drug based on glucagon are summarized in Table 3. This polypeptide with a molecular mass of 3.5 kDa is able to increase the blood glucose concentration. The efficiency of orally administered drug is similar to the efficiency of that with subcutaneous administration.

The carrier synthesized also ensures the transport through the intestine mucous membrane of polypeptides with a higher molecular mass, in particular, the growth hormone with a molecular mass of about 20 kDa (Table 4). The oral administration of the hormone increases its blood

Table 3. Time dependence of the concentrations of glucagon and glucose in blood of rabbits upon administration of 0.5 mg of glucagon

t/min	Subcutaneous injection as a solution		Oral administration of the hydrogel	
	Glucagon (%)*	Glucose/ mg 100 mL^{-1}	Glucagon (%)*	Glucose/ mg 100 mL^{-1}
0	100	107	100	110
5	—	161	—	169
10	130	160	—	215
15	—	169	180	267
30	150	178	140	244
60	100	145	100	177

* Percent of the initial one.

Table 4. Dependence of the concentration of the growth hormone and the glucose concentration in the blood of rabbits upon oral administration of 1.0 mg of the hydrogel-immobilized hormone

t/min	Growth hormone/ ng mL^{-1}	Glucose/ mg 100 mL^{-1}
0	0.17	94
15	8.27	100
30	12.0	148
60	11.4	152
90	13.3	117

concentration, moreover, it enters the blood in the native state, as indicated by the blood glucose concentration. The products of hydrolysis of the growth hormone are known to exhibit insulin-like properties; therefore, the presence of these products in the blood would reduce the glucose concentration.

Thus, the results demonstrate that the polymeric system we synthesized differs in kind from all other known insulin drugs for oral administration: the therapeutic doses of the orally administered drug are similar to injected insulin doses; a correlation exists between the amount of introduced insulin and the level of reduction of the glucose concentration; the preparation exhibits no species specificity, being versatile and active in blood of both healthy individuals and diabetes patients. This provides grounds for some hope for the possibility of using this drug for treatment of diabetes mellitus according to a new, in principle, scheme and opens up prospects for the synthesis of a broad range of polypeptide drugs with enhanced stability against the action of proteolytic enzymes.

Naturally, the successful completion of such many-sided work would be impossible without joint effort of skilled scientists and specialists. The authors are grateful to L. K. Starosel´tseva, E. V. Arzamastseva, A. S. Ametova, and A. G. Goncharova for the assistance in medico-biological and clinical trials of the synthesized preparations.

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