

# Biocompatibility and immunocompatibility of water-soluble polymers based on HPMA

Blanka Říhová \*

*Institute of Microbiology Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic*

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## Abstract

Water-soluble copolymers based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) are investigated as potential carriers of anticancer drugs. For application of such compounds in therapeutic practice their immunogenicity must be checked and copolymers must be found having a structure which would not only be suitable for drug targeting, but would also induce the smallest possible defense reaction in recipient organism. Homopolymer poly(HPMA) with MW around 30 kDa is not recognized as a foreign molecule as no defense reaction against it was recorded. The attachment of side oligopeptide sequences to the HPMA backbone bestows a certain degree of immunogenicity which depends on the composition of the oligopeptidic side chains, dose and route of application, MW and the genotype of immunized individual. HPMA copolymers not only fail to induce a significant immune response against them but they have similar capacity as PEG to dramatically reduce the antibody response against proteins bound to them as a targeting moiety or for the therapeutic purpose. Moreover, the treatment with HPMA-based polymeric drugs evokes in host organism systemic anti-cancer immunity involving both specific and non-specific defense mechanisms of cancer-bearing host.

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## 1. Introduction

The basic mechanisms of biocompatibility of biomaterials are still far from being completely understood despite the fact that knowledge of the principles of compatibility has increased. Water-soluble synthetic polymers based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) studied as carriers for drugs [1–4] are substances foreign for the recipient organism (Fig. 1). Thus, their ability to provoke in the host any kind of the defense reaction has to be carefully examined.

Generally, the molecules that induce an immune response are called antigens/immunogens. Their contact with the cells of the immune system induces adaptive (specific, acquired) defense reaction that leads to the production and secretion of antibodies into the blood and tissue fluids (the humoral

response) and intensive proliferation of specifically activated T lymphocytes that have a regulatory ( $CD4^+$ ; T helper or  $T_H$  lymphocytes) or effector function ( $CD8^+$ ; T cytotoxic or  $T_C$  lymphocytes) in so-called cellular immune response. Innate (natural) immune response involves natural killer (NK) cells and macrophages and their biologically active products. Almost all macromolecules can serve as antigens: proteins, sugars, lipids, phospholipids, glycoproteins, glycolipids, nucleic acids and also synthetic material such as HPMA. The production of antibodies is determined in peripheral blood usually by ELISA or RIA test. Specific cellular response can be assessed *in vitro* as an activation of immunocompetent cells such as human peripheral blood leukocytes (PBL) or mouse splenocytes. The factors important for polymer–drug conjugate design are now rather well established. The polymeric carrier must be biocompatible and immunocompatible, which represents a complex response to the medical material which depends not only on the dose, the route and frequency of administration of foreign material

\* Tel.: +420 241062343; fax: +420 244471286.

E-mail address: [rihova@biomed.cas.cz](mailto:rihova@biomed.cas.cz)

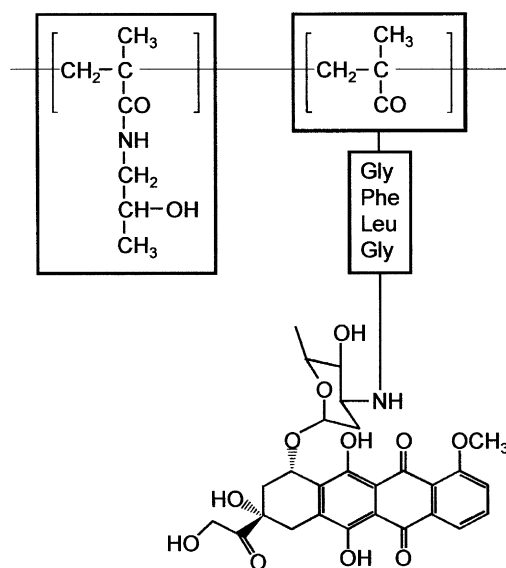


Fig. 1. Doxorubicin-HPMA copolymer, PK1 (FCE 28068).

but also on the genotype (genetic background) of the recipient [5]. Polymers must be readily eliminated, and preferably be biodegradable [4].

The cytotoxic and immunosuppressive drugs which are used for the treatment of tumors, autoimmune diseases, and in transplanted patients are not specific enough in the sense that not only is the pathological process affected by the therapy, but the suppressive activity involves other normal cellular systems, especially the hematopoiesis, gonads, and normal function of mucous membranes of the GI and urinary tract. The side effects, especially after intensive treatment, lead very often to unwanted complications, such as a dramatic increase of sensitivity to different bacterial and viral infections, and are therefore the limiting factors for the use of the most effective substances. This dilemma, i.e., the search for more effective cytotoxic drugs with less cytotoxic general effects, could be best solved by finding a targeting system which would ensure that the drug will reach and act only at the site of the pathological process. Such treatment is called “affinity therapy” and is based on the active concentration of the targeted drug at the pathological sites, which interacts with a particular cell receptor via a specific targeting moiety. The effectiveness of “affinity therapy” depends critically on a suitable carrier system which must remain in the body long enough to carry the drug to the pathological site and must be biocompatible and immunocompatible.

Polymeric conjugates exhibit also significant passive tumor accumulation due to the enhanced permeability and retention (EPR) effect [6,7]. The EPR effect is a consequence of an increased vasopermeability of the tumor vasculature endothelium which is often discontinuous and allows extravasation and accumulation of macromolecules in solid tumor tissue. Absence of tumor lymphatic drainage promotes the retention of the macromolecules. Consequently, the clearance of tumor-accumulated material lar-

ger than 50 kDa is strictly limited. HPMA copolymers are, under physiological conditions, uncharged and easily deformable since they lack any rigid tertiary structure. Both of these properties are known to enhance extravasation, as uncharged and flexible molecules easily pass through an endothelial layer. EPR-mediated targeting was first observed using HPMA copolymer-bound daunomycin [8,9] and later confirmed for HPMA copolymer platinates [10,11] and HPMA-doxorubicin [12–15].

In water-soluble synthetic polymeric delivery systems based on HPMA backbone both the drugs and the targeting moieties (polyclonal and monoclonal antibodies, lectins, carbohydrates, growth hormones, peptides) are bound to the variable oligopeptide side-chains, mostly–Gly-Phe-Leu-Gly-OH [16,17]. After i.v. or i.p. administration, HPMA-based drugs, both non-targeted and targeted, first have to be passively accumulated at the site of the solid tumor by the EPR effect. At the beginning of treatment of a solid tumor, the active targeting is operating only against metastatic cells in the blood-stream. Once passively accumulated at the tumor site, non-targeted and targeted conjugates enter the cells by different mechanisms. Non-targeted conjugates are taken up by non-specific pinocytosis or by other membrane-associated processes which are fast and strictly depending on the physico-chemical character of the conjugate and on the structure of cell plasma membrane. The targeted conjugate is accumulated by receptor-mediated endocytosis the rate of which depends on the ligand (targeting moiety) and target cell receptor [18]. This process is specific and only cells with appropriate receptors are subjected to the cytotoxic action of the targeted polymer-bound drug.

Immunogenicity is already known to be a problem associated with the application of murine antibodies in clinics, either for diagnostic purposes, radiotherapy or in the form of antibody conjugates for chemotherapy (immunotoxins). The human immune system recognizes the rodent antibody as foreign and mounts a HAMA (human anti-mouse) response, giving rise to patient’s antibodies against the rodent monoclonals. HAMA response and response against toxin moiety develops in about 50% patients after a single dose and in about 90% after a second treatment. Subsequent doses of antibody conjugate are often removed from the circulation before they can reach the disease target. It is rather surprising that patients with a functional immune system make anti-immunoglobulin antibodies even when humanized monoclonals are used. Circulating anti-conjugate antibodies can inhibit the efficacy of antibody-targeted drugs by increasing their rate of clearance and/or blocking the binding site on the targeting antibody. Immune reaction against foreign protein might lead to numerous complications, ranging from inactivation of the conjugate by antibodies and/or activated immunocompetent cells to different hypersensitivity reactions. These may range from simple urticaria and culminate in life-threatening anaphylactic reaction. The serious problem is that once the immune reaction begins, the speed of elimination of injected material increases with

repeated applications of antigenic material. In clinical practice this may call for an increase of the injected dose to achieve the required therapeutic effect.

## 2. The humoral (antibody) response against HPMA

The homopolymer of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and its copolymers, which differ in the structure and number of their oligopeptide side chains, were studied for their possible use in human medicine as “polymer therapeutics” [19]. The homopolymer, poly(HPMA), was one of the candidates of blood plasma expander [20,21] and copolymers of HPMA are promising drug carriers. For this reason the homopolymer and the different copolymers were tested for their immunochemical properties and their ability to induce an antibody response.

The homopolymer poly(HPMA) with MW around 30 kDa is not recognized as a foreign molecule as no defense reaction against it was recorded. <sup>14</sup>C-pHPMA showed lack of mitogenicity, hematotoxicity and immunogenicity [22] and when non-radioactive derivative was injected *in vivo*, afferent lymph nodes were not activated [23]. Intraperitoneal administration of an alum precipitate in doses of 1–100 µg did not induce the formation of a detectable level of antibodies in any of the five inbred strains of mice tested; C3H, C57L/J, C57BL/10, A/J, Balb/c [24,25] (Table 1). In this respect the poly(HPMA) resembles the synthetic polymers of one or two alpha amino acids, which also were proven to be non-immunogenic in mice [26]. It appears that an important attribute for the immunogenicity of an injected material is a certain degree of its heterogeneity as more complex polymers are more immunogenic. The defense reaction against them depends on their size and shape, electric charge, composition, dose, the way of introduction and, last but not least, on the inherited ability of the host to respond to a foreign material.

The attachment of side oligopeptidic sequences to the HPMA backbone bestows a certain degree of immunogenicity to such a copolymer molecule. We compared the immunogenicity and mitogenic activity of copolymers differing in oligopeptide side chains (-Gly-Gly-OH; -Acap-Phe-OH; -Acap-Leu-HMDA and -Gly-Phe-Tyr-OH) or in

their content (1%, 3.5% and 8.4% mole of -Gly-Gly-OH). Immunization with copolymers led to the antibody response which, however, was very weak. On the average, the titer of the antibodies was lower by four orders of magnitude than that of antibodies against the reference bovine gamma globulin (BGG). This indicates a very low immunogenicity of the tested compounds. As the immune response strictly depends on the amount of injected antigen, mice were immunized with different doses of copolymers varying from 1 to 100 µg. In addition, different vehicles were selected, i.e., the material was applied as a solute, as an alum precipitate or injected in complete/incomplete Freund's adjuvant. All copolymers were able to induce antibody formation in the whole range of tested doses. The highest levels were detected at a dose of 10 µg. Doses of 1 µg but also of 100 µg mostly led to a lower antibody formation [24,25]. It should be stressed that the dose leading to the strongest reaction may differ considerably for different types of antigens, especially synthetic polymers, reaching sometimes even strikingly low values of about 0.01 µg.

An important factor for the intensity of the antibody response might be the composition of the oligopeptidic side chains. It was noteworthy that the sequence -Gly-Gly-OH induced higher antibody levels than the sequences -Acap-Leu-HMDA, -Acap-Phe-OH, or even the sequence -Gly-Phe-Tyr-OH [27]. This result was interesting and rather unexpected as it was repeatedly shown that the presence of aromatic side chains in the antigen increases its immunogenicity [26].

The number of side chains, in the tested range, was unimportant for the immunogenicity. Copolymers differing in the content of side chains -Gly-Gly-OH (1.0%; 3.5% and 8.4% mole) induce a very similar immune response in mice. A very interesting finding is the immunogenicity of the copolymer with 1.0% content of -Gly-Gly-OH side chains where, for every molecule of the copolymer, there are only two side chains which are the main antigenic determinants or epitopes [27] (Table 2).

If the end of the oligopeptidic side chain was modified with a substance that behaves as a hapten (DNP, arsanilic acid, fluorescein isothiocyanate) a significant immune response against such a conjugate was detected by PFC, ELISA and haemagglutination test. Most of these antibodies were aimed against the modifying haptenic group and only low amount against the side oligopeptide sequences of the carrier. This means that the main antigenic determinant (epitope) of the copolymer molecule is a derivatized oligopeptide side chain which also determines the specificity of formed antibodies. The overall level of antibody against the copolymer derivative depends on the immunochemical characteristics of the modifying, drug-resembling hapten. For instance, the reaction against the fluorescein isothiocyanate group containing copolymer was ten times higher than to the copolymers containing *p*-azophenylarsonate or DNP group. It seems that the antibodies may arise also against poly(HPMA) itself. These chains, though antigenic, i.e., able to react with the once formed antibodies, are

Table 1  
Immunogenicity of HPMA copolymers in A/J mice (ELISA)

| Antigen                    | Antigen (µg)                |    |    |
|----------------------------|-----------------------------|----|----|
|                            | 100                         | 10 | 1  |
| P <sup>a</sup> -Gly-Gly-OH | 6 <sup>b</sup>              | 7  | 6  |
| P-Acap-Phe-OH              | 4                           | 6  | 6  |
| P-Acap-Leu-HMDA            | 4                           | 6  | 4  |
| P-Gly-Phe-Tyr-OH           | 4                           | 4  | 3  |
| P-Gly-Phe-Leu-Gly-OH       | 5                           | 4  | 5  |
| Poly(HPMA)                 | No antibodies were detected |    |    |
| Bovine γ-globulin          | 28                          | 22 | 13 |

<sup>a</sup> P = HPMA.

<sup>b</sup> log<sub>2</sub> of serum dilution.

Table 2  
Influence of side oligopeptide chains on the immunogenicity of HPMA in different inbred strains of mice

| Antigen (copolymer)        | Content of side chain (%mol) | Antibody production in inbred strains of mice <sup>a</sup> |                         |                            |                                       |                         |
|----------------------------|------------------------------|--|-------------------------|----------------------------|---------------------------------------|-------------------------|
|                            |                              | C57L/J<br>H-2 <sup>b</sup>                                 | A/J<br>H-2 <sup>a</sup> | Balb/c<br>H-2 <sup>d</sup> | B/10 <sup>b</sup><br>H-2 <sup>b</sup> | C3H<br>H-2 <sup>k</sup> |
| P <sup>c</sup> -Gly-Gly-OH | 1.0                          | 7 <sup>d</sup>   | 8                       | 8                          | 9                                     | 5                       |
| P-Gly-Gly-OH               | 3.5                          | 6  | 6                       | 7                          | 8                                     | 5                       |
| P-Gly-Gly-OH               | 8.4                          | 7  | 7                       | 5                          | 7                                     | 4                       |
| P-Acap-Phe-OH              | 1.8                          | 2  | 6                       | 5                          | 5                                     | 4                       |
| P-Gly-Phe-Tyr-OH           | 2.3                          | 2  | 4                       | 4                          | 4                                     | 3                       |
| Poly(HPMA)                 | –                            | No antibodies were detected                                |                         |                            |                                       |                         |

<sup>a</sup> Dose = 10 µg of antigen in CFA.

<sup>b</sup> B/10 ... C57BL/10.

<sup>c</sup> P ... HPMA.

<sup>d</sup> Antibody concentration is expressed as log<sub>2</sub> serum dilution.

nevertheless not immunogenic, which means that they are not able by themselves to induce antibody response.

The dependence of the immune reaction on the molecular weight of the copolymer was studied in experiments where a copolymer with -Acap-Leu-HMDA-ARS side chains was used as an antigen and the immune reaction was expressed by the numbers of plaque (antibody) forming cells. Compared with the fraction of molecular weight 5000, fraction with molecular weight between 150,000 and 200,000 brought about a 2- to 5-fold increase in the number of antibody-forming cells in the spleen [24,28]. The most likely explanation is that the low molecular weight fractions are rapidly removed from the blood circulation, which decreases the chance of immunocompetent cells to come into contact with tested foreign material and react to it.

It is known that the genotype of an immunized individual may affect decisively the development of the immune response. The ability to respond to a particular antigenic determinant is linked to the major histocompatibility locus (MHC; H-2 in mice). Thus, antibody formation against poly(HPMA) and HPMA copolymers was studied in inbred mouse strains of four different haplotypes (Balb/c = H-2<sup>d</sup>; C57BL/ScSn = H-2<sup>b</sup>; C3H = H-2<sup>k</sup> and A/J = H-2<sup>a</sup>) and in two strains of the same haplotype but differing in the remaining genetic background (strains C57BL/10ScSn and C57L/J). Antigens were administered three times at two-week intervals in three different doses, 1, 10 and 100 µg as a solute as well as in complete Freund's adjuvant (CFA). Antibodies were assayed one and two weeks after the last immunization by haemagglutination and ELISA assay. The results revealed some differences in antibody response of the immunized strains. These differences, however, were not significant and cannot be taken as proof of the effect of the H-2 haplotype on the course of the immune response to the studied copolymers [24,25,27] (Table 2).

Before introducing polymeric prodrugs based on HPMA into clinical trials I and II [29] we carefully tested both non-targeted (PK1; Fig. 2) and galactosamine-targeted (PK2; Fig. 3) derivatives for their immunogenicity. Both conjugates contained doxorubicin bound to the polymer backbone via glycyphenylalanylleucylglycine side

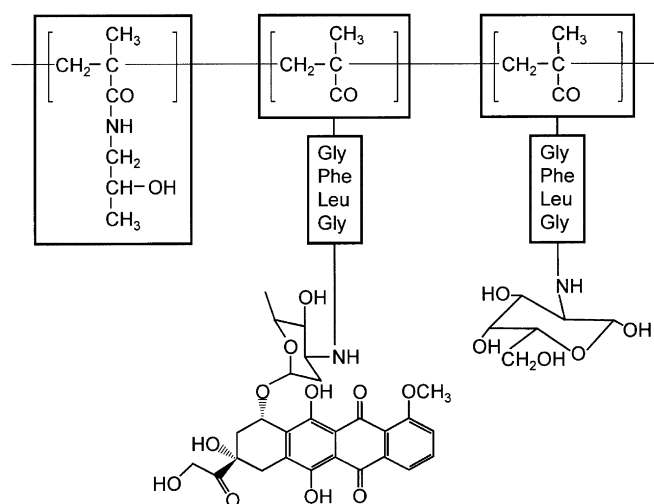


Fig. 2. Doxorubicin-HPMA copolymer-galactosamine, PK2 (FCE 28069).

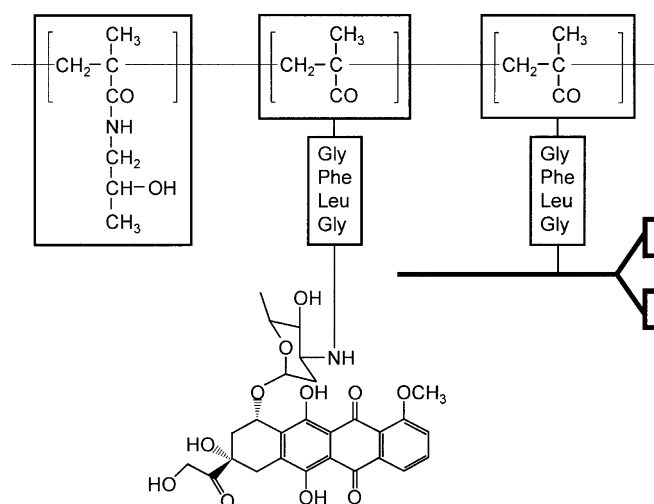


Fig. 3. Doxorubicin-HPMA copolymer-HuIg.

chain. Two different inbred strains of mice (T<sub>h</sub>1-prone A/J and T<sub>h</sub>2-prone C57BL/10) were injected and the ability of synthetic polymers to induce an antibody response was

Table 3  
Immunogenicity of PK1 and PK2 (ELISA)

| Sample (structure)      | Dose                        |      |        | Route of application |          |
|-------------------------|-----------------------------|------|--------|----------------------|----------|
|                         | Copolymer ( $\mu\text{g}$ ) | DOX  |        | A/J                  | C57BL/10 |
| P-GFLG-DOX <sup>a</sup> | 300                         | 25.5 | i.v.   | 7 <sup>b</sup>       | 7        |
|                         |                             |      | s.c.   | 7                    | 8        |
|                         |                             |      | Orally | 7                    | 8        |
| /                       | 100                         | 8.5  | i.v.   | 7                    | 9        |
|                         |                             |      | s.c.   | 6                    | 9        |
|                         |                             |      | Orally | 7                    | 8        |
| P \ GFLG-gal            | 300                         | 21.9 | i.v.   | 8                    | 7        |
|                         |                             |      | s.c.   | 7                    | 8        |
|                         |                             |      | Orally | 6                    | 5        |
|                         | 100                         | 7.3  | i.v.   | 6                    | 7        |
|                         |                             |      | s.c.   | 5                    | 6        |
|                         |                             |      | Orally | 6                    | 5        |
| Control                 | –                           | –    | –      | 5                    | 6        |

<sup>a</sup> PK1 ... doxorubicin-HPMA; FCE28068.

<sup>b</sup>  $\log_2$  of serum dilution.

<sup>c</sup> PK2 ... doxorubicin-HPMA-galactosamine; FCE 28069.

determined in the serum using the enzyme-linked immunoassay (ELISA). Experimental mice were injected five times by a dose of 10–300  $\mu\text{g}$ . Both samples were applied only as a solution as only this form might be used for the medical treatment. Serum antibody level was tested on day 3 and on day 6 to evaluate the kinetics of the antibody formation. Control groups of animals which were not immunized showed a natural titer of antibodies against the test HPMA copolymer conjugates which varied between 5 and 6  $\log_2$  of serum dilution, with no detectable differences between A/J and C57BL/10 mice. After multiple immunizations of animals with PK1 and PK2 conjugates the increase in the antibody production did not exceed one or two dilutions of sera (Table 3). This means that the immunogenicity of the injected samples was very low. Intravenous, subcutaneous and oral immunization produced similar antibody titers [30].

At the end of this chapter it is important to stress that the inability of a material to elicit a significant formation of antibodies still does not mean that the compound is entirely inert from the immunological point of view.

### 3. The cellular immune response against HPMA

To this aim we have investigated the mitogenic activity of HPMA copolymers differing in the content and composition of oligopeptide side chains. The mitogenic activity was determined *in vitro* by measuring incorporation of <sup>3</sup>H-thymidine into DNA of naive or presensitized spleen cells of C57L/J mice after primary and secondary *in vitro* stimulation with various doses (100, 10, 1 and 0.1  $\mu\text{g}$ ) of copolymers. Neither the homopolymer poly(HPMA) nor the copolymers with oligopeptidic side sequences -Gly-Gly-OH, -Gly-Phe-Tyr-OH, -Acap-Phe-OH, or -Acap-Leu-HMDA induced proliferation of mouse T and B lymphocytes [24,25,27]. The same result was obtained in

the study of secondary mitogenic stimulation in which the reaction was investigated of spleen cells taken 14 days after *in vivo* primary stimulation with 10  $\mu\text{g}$  of the respective polymer (copolymer) [27].

### 4. Complement activation

The complement system is one of the major effector mechanisms of humoral immunity and is also an important effector mechanism of innate immunity. It consists of several serum and cell surface proteins that are normally inactive; they are activated only under particular conditions to generate products that mediate various effector functions of complement. There are three major pathways of complement activation. The classical pathway, so called because it was discovered first, uses a plasma protein called C1 to detect IgM, IgG1, or IgG3 antibodies bound to the surface of a microbe or other structures. The alternative pathway is triggered by direct recognition of certain microbial surface structures and is thus a component of innate immunity. The lectin pathway is triggered by a plasma protein called mannose-binding lectin (MBL), which recognizes terminal mannose residues on microbial glycoproteins and glycolipids. The most important consequences of complement activation result from the pharmacological effect of some of the activated components. Determination of the potential to activate complement can be used as one criterion in testing the biocompatibility of various synthetic polymers or copolymers.

While complement activation as a reaction to foreign substances is certainly beneficial, if not essential, stimulation under certain circumstances may contribute to, or even evoke a pathological reaction resulting in tissue injury and diseases. Activated complement components have been reported to effect the regulation of the humoral and cell-mediated immune responses such as mitogen-induced

lymphocyte proliferation, lymphokine production, macrophage activation and antibody response. Some polymers are known to cause activation of complement. This holds especially for polycations whereas polyanions often inactivate C1 or C2 components effective in the classical pathway of complement activation. Since soluble HPMA copolymers are a combination of synthetic polymeric chains with oligopeptide side chains, their structure suggested that they might influence the complement system. Thus, we studied the effect of the structure of HPMA copolymers differing in the structure and content of oligopeptide side chains on the classical and alternative pathway of porcine complement *in vitro*. It was found that the HPMA homopolymer, as well as copolymers containing oligopeptide sequences terminated in carboxylic groups, amino groups, aromatic units or puromycin have no prominent effect on the porcine complement system *in vitro*. Inhibition of both pathways of the complement system occurred only at a very high concentration (20 mg/ml) highly exceeding the dose considered for therapeutic purposes. Lower concentrations (2 and 0.2 mg/ml) did not activate either the classical or the alternative complement activation pathway [31].

### 5. Long-term application

Phagocytic cells, macrophages and neutrophils, developed during phylogeny to remove from the organism potentially foreign materials, originally microbes. They can be easily overloaded with a large dose of foreign synthetic and only partially degradable material, which may induce short or long-lasting blockage of the body phagocytic system. As the phagocytic cells are antigen presenting cells (APC) that play an important role in presenting antigens derived from endocytosed material their overloading/blocking may easily induce a collapse of the defense response. In the effector phase of cell-mediated immunity, differentiated effector T cells recognize foreign antigens on phagocytes and activate macrophages to destroy them. To test the functional ability and the immune capacity of the mice undergoing long-lasting injection of synthetic material such as HPMA the antibody response to protein and microbial antigens has been evaluated. Theoretically, the decrease of the ability to form antibodies could be associated with limited antigen-presentation function of APC overloaded with foreign material. The main source of the most immunocompetent cells is the bone marrow which contains a heterogeneous population of hematopoietic and lymphopoietic precursors. These are highly sensitive to toxic substances. After transfer into sublethally irradiated recipients' stem cells, especially hematopoietic precursors, form colonies in different tissues which in spleen can be easily enumerated as colony-forming unit spleen (CFU-s). We have injected two inbred strains of mice, A/J and C57BL/10, with a total dose of 2 g/kg of HPMA copolymer containing 6.3% oligomeric side chains -Gly-Phe-Leu-Gly-OH with molecular weight of 24 kDa. The copolymer was administered to inbred mice intraperitone-

ally three times per week in a dose of 2 mg/0.5 ml for two months. Our data indicate that (a) the activation of classical and alternative complement pathway was not affected, (b) phagocytic function of peritoneal macrophages was not changed, (c) antigen-presenting function on APC was not destroyed and (d) bone marrow stem cells colony-forming capacity and (e) the numbers of T and B lymphocytes in the peripheral blood of experimental animals were comparable with controls. When the organs of the experimental animals were examined histologically no specific pathological findings were observed which could be attributed to copolymer administration. As the doses used were much higher than those that would be used in clinical practice, copolymers HPMA were considered as biocompatible, non-toxic suitable carriers for drugs [32].

### 6. Decreased immunogenicity of proteins conjugated to HPMA

The major obstacle for long-lasting therapy with pharmacologically active proteins, such as enzymes, antibodies or immunotoxins, is their immunogenicity. The immune reaction of the host might lead to serious complications, ranging from inactivation of the biologically active macromolecule by antibodies and/or activated immunocompetent cells, to different hypersensitivity reactions. Even when the immune response does not culminate to any dramatic manifestations, the simple fact that it results in a rapid clearance of the pharmacologically active protein can cause many difficulties. It is a rule that once the immune reaction begins, the speed of elimination increases with the repeated applications of the antigenic material. It usually calls for an increase of the injected dose to achieve the required therapeutic effect. Abuchowski and co-workers were the first to develop a method for the reduction of the immunogenicity of therapeutic proteins by their covalent binding to methoxy poly(ethylene glycol) (PEG) [33,34]. This method, now called "pegylation" is widely used for the reduction of the immunogenicity of proteins or to reduce the non-specific uptake of liposome/nanoparticle-entrapped drugs and other therapeutic agents.

HPMA copolymers not only fail to induce a significant immune response against them but they have similar capability as PEG to dramatically reduce the antibody response against proteins bound to them as a targeting moiety or for the therapeutic purpose. We and others compared the immunogenicity of the original proteins and HPMA copolymer-modified rabbit IgG, human IgG, bovine IgG, transferrin, human serum albumin (HSA), bovine seminal RNase, chymotrypsin, superoxide dismutase (SOD) and mouse anti-Thy 1.2 monoclonal antibody. Because of the known importance of the genetic background of mice on the immune response, two strains of mice, high IgG antibody responders A/J and low IgG antibody responders C57BL/10 were used. To exaggerate any differences in immunogenicity of the model proteins, antigen was usually given in a complete Freund's adjuvant (CFA). For a more

Table 4  
Immunogenicity of HPMA-rIgG<sup>a</sup>

| Structure                                 | Antibody titer          | rIgG<br>HPMA-rIgG |
|---|-------------------------|-------------------|
| GFLG-doxorubicin<br>/<br>p <sup>b</sup> \ | 2.1 × 10 <sup>6</sup>   | 123               |
| GFLG-rIgG                                 |                         |                   |
| GLG-doxorubicin<br>/<br>P \               | 1.0 × 10 <sup>6</sup>   | 260               |
| GLG-rIgG                                  |                         |                   |
| P-GFLG-rIgG                               | 3.7 × 10 <sup>6</sup>   | 70                |
| rIgG                                      | 260.0 × 10 <sup>6</sup> | –                 |

<sup>a</sup> rIgG ... rabbit IgG.

<sup>b</sup> P ... HPMA.

clinically relevant approach experiments were also carried out with antigen administered in phosphate-buffered saline (PBS). The immunogenicity of all tested proteins was greatly reduced after their conjugation to the HPMA copolymer carrier [25,35,36]. The decrease in the antibody response was even higher if compared with conjugation to the PEG<sub>5000</sub> [37]. Depending on the tested system, the titers after immunization with HPMA copolymer-modified conjugates were 70–260 times lower compared with the parent protein (Table 4). Such results confirm the hypothesis that antigenic epitopes of proteins bound to the HPMA copolymer carrier are hidden and unavailable for both immunization and detection. Moreover, HPMA copolymer-bound proteins are probably protected from enzymatic degradation which is essential for the antigen processing and presentation. Decrease in the immunogenicity of chymotrypsin after its modification with semitelechelic poly(HPMA) was related to the molecular weight of synthetic carrier. While conjugation to low-molecular weight poly(HPMA) of a MW = 2940 Da does not significantly reduce the immunogenicity of chymotrypsin indicating that the polymer shell on the protein surface is not sufficiently thick to prevent the enzyme from being recognized by the cells of the immune system, a decrease in chymotrypsin immunogenicity was already detected in conjugates modified by polymer of MW = 7250 and 19,470 Da [38]. This is in accordance with the results of several other authors who used pegylation [39].

Considerably lower levels of anti-SOD (anti-superoxide dismutase) antibodies were detected in mice immunized with poly(HPMA)-SOD or HPMA-SOD copolymer carrier instead of non-modified SOD. The antibody raised against the “classical” form of the PHPMA-SOD conjugate were significantly higher than the levels formed against a “star-like” form of poly(HPMA)-SOD conjugate. In the “classical” conjugate the SOD molecules react with the HPMA copolymer precursor containing many reactive ONp groups randomly distributed along the polymeric chain. In the

“star-like” conjugate the SOD molecules react with the semitelechelic poly(HPMA) copolymer precursor containing only one reactive NHS group at the end of the polymeric backbone. The reactivity of once formed anti-SOD antibodies with both types of conjugates, i.e., “classical” and “star-like” is low which suggests either (a) the loss of the original SOD immunodominant epitopes, (b) the creation of new epitopes during the conjugation process or that (c) immunodominant epitopes are hidden and not available for the antigen–antibody reaction. Similarly, the antibodies raised against both types of HPMA based-SOD conjugates react only to a very limited extent with original non-modified SOD. Higher titers detected with antibodies raised against HPMA-SOD conjugates and homologous antigens, i.e., with “classical” and “star-like” polymer-SOD support the idea that new polymer-SOD-specific immunodominant epitopes are created during conjugation. Different titers in which anti-SOD antibodies react with PHPMA-SOD and with SOD further suggest that the binding of the protein to the polymeric carrier also affects its presentation and/or recognition by antigen-presenting cells (APC) and, consequently, the activation of Th lymphocytes [40]. A decrease of SOD immunogenicity after binding to PEG was reported by Saifer et al. [41].

We have demonstrated also in human patients that a targeting protein moiety attached to a HPMA copolymer carrier loses its ability to induce an antibody formation. Cancer patients were treated five times with doxorubicin-HPMA copolymer-bound human IgG and the presence of serum anti-IgG antibodies was checked by ELISA at 2-week intervals. Up to three weeks after the fifth application of the conjugate, i.e., 7 months after the first treatment, no traces of antibodies against allogeneic human immunoglobulin were detected in the patient’s serum [3,42].

The mechanism by which the immunogenicity of the proteins is reduced after their conjugation to different polymeric carriers has not yet been fully clarified. Non-immunogenic HPMA copolymer might simply masks the antigenic determinants of the targeting protein by sterically hindering their accessibility, a mechanism ascribed to PEG reduction of protein immunogenicity following conjugation. Alternatively, up to 50% decrease observed in proteolytic degradation of antibody bound to HPMA copolymer compared to parent antibody [43] may interfere with normal pathways of intracellular protein degradation necessary for antigen processing and presentation, resulting in decreased immune response. Antigen processing is the intracellular conversion of protein antigens derived from the extracellular space or the cytosol into peptides and loading of these peptides onto major histocompatibility complex (MHC) molecules for display to T lymphocytes. The display of peptides bound by MHC molecules on the surface of an APC permits specific recognition by T cell receptors and activation of T cells.

Oupicky et al. [44] compared long-circulating vectors, polyplexes containing DNA and poly-L-lysine or polyethyl-

eneimine, surface modified with either monovalent polyethylene glycol or multivalent copolymers of *N*-(2-hydroxypropyl)methacrylamide correlating their biophysical properties with their distribution following intravenous injection. In contrast to coating with monovalent PEG, coating with multivalent PHPMA results in improved circulatory properties for all polyplexes examined, irrespective of the type of polycation used and its molecular weight. The improvement in circulatory properties of the PHPMA coated polyplexes is not primarily due to their negative charge or the chemical nature of the coating polymer but instead is a consequence of the lateral stabilization provided by the coating polymer. It was shown that efficient lateral stabilization and subsequent prolonged circulation of the coated polyplexes demands the use of PHPMA with minimal molecular weight of 30 kDa and that the concentration of PHPMA plays a vital role in enabling prolonged circulation.

## 7. Decrease side-toxicity of HPMA-bound drugs

Conjugation of drugs to HPMA copolymer carrier considerably reduces their side toxicity. It applies for heart and liver toxicity and myelotoxicity of anthracyclines [1,4,45–49], dark toxicity of photosensitizer chlorin  $e_6$  [50] and thymus and kidney toxicity of cyclosporin A [51,52]. Our experimental data further suggest that the treatment with HPMA-based drugs represents not only an efficient and safe chemotherapy but also a kind of immunotherapy as it simultaneously activates the immune system of the host.

### 7.1. Immunomodulation

The immune response can be influenced in two ways. Enhancement of the immune response is called immunopotential and its down-regulation immunosuppression. Compounds that are capable of interacting with the immune system to up-regulate or down-regulate the defense response are called immunomodulators or biological response modifiers. Immunomodulation involves the cellular level and the molecular level, i.e., induction of cytokines and chemokines. Major effector cells of the immune system that kill potentially dangerous cells (infected or tumor cells) are activated macrophages, natural killer (NK) cells, specific cytotoxic T lymphocytes (CTL) and lymphokine-activated killers (LAK).

Whether certain compounds enhance or suppress immune response depends on a number of factors including dose, route and time of administration, the mechanism of action of the immunomodulator and the site of its activity. The use of biological response modifiers for controlled immunomodulation requires precise and detailed knowledge of their target(s) to avoid unwanted stimulation/suppression of particular cell compartments. This is task number one, as biological responder modifiers can often act both as immunostimulators and as immunosuppressants [53].

Immunopotential can be defined as a process that directly enhances the activity of one or more components of the complex immunoregulatory network. There is no doubt that neither the homopolymer poly(HPMA) nor the HPMA copolymer induces a significant defense reaction against themselves or against protein moieties that are bound to them. However, they are not entirely inert from the immunomodulation point of view. We have seen *in vivo* that they somehow influence the immune response induced in the host by foreign antigens. *In vitro* we have observed a significant immunostimulatory effect of HPMA containing -Gly-Phe-Leu-Gly-OH tetrapeptide side-chains on the spontaneous proliferation of human peripheral blood mononuclear cells (PBMC) and on mouse splenocytes. The pro-proliferative effect of the copolymer was considerably greater than the effect of the homopolymer, poly(HPMA), alone. Terminating the tetrapeptide side-chains with human serum albumin (HSA), bovine gamma globulin (BGG) or doxorubicin reduced the stimulatory effect of the polymeric carrier. Supernatants from a cell culture of splenocytes exposed for 17–72 h to doxorubicin, HPMA copolymer-bound doxorubicin, i.e., PK1 or HPMA copolymer without drug, stimulated the spontaneous proliferation of C57BL/6 or Balb/c spleen cells in the following order: HPMA copolymer > homopolymer poly(HPMA) > HPMA copolymer-bound doxorubicin > doxorubicin. Balb/c splenocytes (TH<sub>2</sub>-prone mice inbred strain producing mainly IL-4) are more sensitive to the effect of released growth factors than C57BL/10 splenocytes (TH<sub>1</sub>-prone inbred strain producing mainly IFN- $\gamma$ ) and the amount of growth factors is higher after 72 h of incubation (Table 5). There is either a direct involvement of the tested compounds in the cell surface proliferation signaling or the cells, after close contact with them, release stimulatory factors such as cytokines which induce resting cells to proliferate [37].

Immunomodulating activity of HPMA derivatives was *in vivo* confirmed by stimulation of the proliferation of immunocompetent T and B cells in regional lymph nodes. Mice were injected intraperitoneally three times on three consecutive days with therapeutic doses of doxorubicin,

Table 5  
Cytokines in supernatants from cell cultures exposed to poly(HPMA) or HPMA copolymer

| Sample (structure) | Concentration ( $\mu\text{g/ml}$ ) | Inbred strain of mice         |      |               |                               |
|--------------------|------------------------------------|-------------------------------|------|---------------|-------------------------------|
|                    |                                    | Balb/c                        |      | C57BL/6       |                               |
|                    |                                    | Th <sub>2</sub> -prone strain | IL-4 | IFN- $\gamma$ | Th <sub>1</sub> -prone strain |
|                    |                                    |                               |      | IL-4          | IFN- $\gamma$                 |
| Poly(HPMA)         | 207                                | 10 <sup>a</sup>               | 48   | 9             | 106                           |
|                    | 69                                 | 51                            | 50   | 8             | 131                           |
|                    | 23                                 | 158                           | 53   | 11            | 217                           |
| HPMA copolymer     | 207                                | 6                             | 11   | 1             | 4                             |
|                    | 69                                 | 80                            | 41   | 9             | 129                           |
|                    | 23                                 | 206                           | 55   | 9             | 187                           |

<sup>a</sup> In pg/ml.

poly(HPMA) homopolymer or HPMA copolymer containing -Gly-Phe-Leu-Gly-OH side chains without drug or with HPMA copolymer-bound doxorubicin (PK1). They were killed on day 7 and the proliferation of regional lymph node cells (LNC) was compared after their *in vitro* stimulation using concanavalin A (proliferation of T lymphocytes) or lipopolysaccharide (LPS; proliferation of B lymphocytes). The proliferation was evaluated either by MTT test or by incorporation of <sup>3</sup>H-thymidine. Quite clearly, injection of PK1 stimulated both T and B cells in regional lymph nodes [37].

### 7.2. Protection of the defense mechanisms of the host by injection with HPMA copolymer-bound doxorubicin

We have observed a substantial difference in the appearance and aggressivity of a transplantable tumor, mouse T cell lymphoma EL4, when injected into control mice or into mice pretreated by five daily injections of cyclosporin A (total dose 135 mg/kg), doxorubicin (total dose 12.5 mg/kg) or of HPMA copolymer-bound doxorubicin (PK1; total dose 25 mg/kg). The immunoprotection by polymer-bound drug was observed over the whole range ( $1 \times 10^4$ – $1 \times 10^6$ ) of injected doses of EL4 cells. While pretreatment with the free drug always accelerated the appearance and increased the aggressivity of the experimental tumor, tumor growth in mice injected with doxorubicin bound to HPMA copolymer carrier was comparable to the non-treated controls. Notably, not only immunoprotection, but also a limited immunomobilization of host defense mechanisms was observed in mice pretreated with HPMA copolymer-bound doxorubicin. Mice pretreated with HPMA copolymer-bound doxorubicin and injected with up to  $1 \times 10^2$  tumor cells did not develop tumors, which appeared regularly in controls and in doxorubicin-treated mice [37,54].

### 7.3. Activation of natural killer (NK) cells and macrophages

Natural killer (NK) cells are large granular lymphocytes (LGLs) with numerous cytoplasmic granules that express cytolytic activity without prior antigenic stimulation and are thought to contribute to the immunity against viruses and surveillance against neoplastic transformation. There are a number of substances, including synthetic ones, able to increase NK and LAK activity for several days. In our study we have determined NK activity in peripheral blood and spleen of athymic nu/nu CD-1 mice subcutaneously heterotransplanted with human colorectal carcinoma SW 620 or in the mouse Thy 1,2-transfected SW 620 (SW 620/T) line and in C3H/HeN mice transplanted with syngeneic B cell lymphoma 38C13. When the tumors developed (7 days after transplantation), the mice were treated i.p. five times at 2-day intervals with HPMA copolymer-bound doxorubicin (PK1; total dose of doxorubicin was 25 mg/kg) or with free doxorubicin (total dose 7.5 mg/kg). NK activity was detected 2 days after termination of the treat-

ment (i.e., on day 17 of the experiment) using the standard method of YAC1 cell killing. The data obtained with the SW620, SW620/T and 38C13 cancer cell lines confirmed our original observation with T cell lymphoma cell line EL4. The impairment of NK activity, observed after injection with free drug, is not observed after treatment with the drug bound to the HPMA copolymer carrier. Most notably, not only was impairment not observed, but also a significant increase in the NK activity in the spleen of nu/nu CD-1 mice bearing the SW 620/T tumor and treated with PK1 was clear if compared with mice treated with classical drugs or non-treated controls. The data suggest a role for NK cells as a part of the effector mechanism immunomodulated by the injection of HPMA copolymer-bound drug [37].

We had a similar experience with human patients suffering from an advanced generalized breast carcinoma. Before the treatment with Dox-HPMA-HuIg, the activity of NK and LAK cells in the patients was comparable or lower to that in healthy donors. Seventy-two hours after the first three treatments we observed an activation of NK cells (target cell line K 562) and LAK cells (target cell lines Daudi and Raji) in patient's peripheral blood. Interestingly, activated NK and LAK cells were detectable in the peripheral blood up to 19 days after the first treatment, up to 14 days after the second treatment and only up to 7 days after the third treatment [3,42]. No comparable activation of NK cells was observed in peripheral blood after the fourth and fifth treatment, when even depressed natural killer activity was seen. Similarly, while an increase in LAK activity has been obvious after the second and third treatment, no such increase was recorded after the fourth and fifth administration of macromolecular therapeutic (Tables 6 and 7). There are at least two explanations for such a result. Either multiple injection of macromolecular therapeutics somehow over stimulated NK and LAK cells resulting in temporary rest similarly as it is known for T and B immunocompetent cells, or activated cells moved from the peripheral blood, where they were determined, to the site of the pathological process [3,37].

We have also tested the activation of macrophages *in vivo* after intraperitoneal injection with doxorubicin bound to HPMA copolymer carrier (PK1) or only with

Table 6  
Activity of NK cells in human patients

| Application   | Day | No. 1           | No. 2 | No. 3 | No. 4 |
|---------------|-----|-----------------|-------|-------|-------|
| Ist           | 0   | 24 <sup>a</sup> | 23    | 9     | 11    |
|               | 3   | 45              | 21    | 19    | 33    |
| IIInd         | 0   | 22              | 10    | 10    | 11    |
|               | 3   | 27              | 25    | 16    | 12    |
| IIIrd         | 0   | 23              | 10    | 8     | 13    |
|               | 3   | 42              | 12    | 6     | 18    |
| Healthy donor |     | 24              |       |       |       |

<sup>a</sup> NK ... % of K562 cell line killing.

Table 7  
Activity of LAK cells in human patients

| Application   | Day | No. 1           | No. 2 | No. 3 | No. 4 |
|---------------|-----|-----------------|-------|-------|-------|
| Ist           | 0   | N.D.            | 25    | 11    | 4     |
|               | 3   | N.D.            | 17    | 15    | 14    |
| IIInd         | 0   | 20 <sup>a</sup> | 14    | 17    | 18    |
|               | 3   | 40              | 18    | 16    | 15    |
| IIIrd         | 0   | 17              | 16    | 13    | 8     |
|               | 3   | 50              | 20    | 5     | 20    |
| Healthy donor |     | 24              |       |       |       |

<sup>a</sup> LAK ... % of Daudi cell line killing.

polymeric carrier, i.e., *N*-(2-hydroxypropyl)methacrylamide copolymer containing a -Gly-Phe-Leu-Gly-OH side-chain without a conjugated cytostatics. Activation was detected *in vitro* as the production of nitric oxide by peritoneal exudate cells (PEC) exposed to LPS and IFN- $\gamma$ . While the injection of PK1 was not followed by a substantial production of NO, injection of HPMA copolymer carrier without the drug induced a significant release of NO by PEC that could be detected in cell culture after 24 and 72 h [37].

#### 7.4. Systemic anti-cancer immunity induced in mice cured by the treatment with HPMA-based non-targeted or antibody-targeted macromolecular therapeutics

Non-targeted or antibody-targeted HPMA copolymer-bound doxorubicin represents a very efficient treatment of experimental cancer [1,2,4,45,48,55–57]. Moreover, the treatment evokes systemic anti-cancer immunity in host organism, as up to 80% of mice cured of the tumor and re-transplanted with a lethal dose of the same cancer cells survive without any additional treatment [15,42,54,56,58]. We have documented that such an immunomobilization involves both specific and non-specific defense mechanisms of the cancer-bearing organism [58]. These data justify the hypothesis that HPMA copolymer-bound drugs, unlike free drugs, have both cytostatic and immunomobilizing activity operating at different times after the treatment. We suppose that immediately after the injection, due to the high level of the drug in the body, the main activity of the polymeric conjugate is cytotoxic and cytostatic. Later on, the long-term circulating polymer-bound drug [1,37,59,60], at concentration lower than its minimal inhibitory value, may stimulate the defense mechanisms of the host similarly as shown *in vitro* for free drugs and some antibiotics [53]. The very rapid elimination of free drugs from the bloodstream usually prevents a comparable stimulation *in vivo*. A certain immunostimulatory activity of HPMA copolymer-bound daunomycin has already been documented in our first paper dealing with the side-effects of polymer-bound drugs. Daunomycin injected i.v. or i.p. in the form of a conjugate with HPMA copolymer was not myelotoxic, but somehow stimulated bone marrow stem cells, leading to an increase in the number of colony-forming unit-spleen (CFU-s) [45]. It is rather a new observation that stress proteins such as

HSP70, which are released during necrotic but not during the apoptotic cell death of cancer cells, stimulate immature dendritic cells in lymph nodes and spleen and elicit a potent pro-inflammatory immune response in human monocytes [61]. Dendritic cells, which often infiltrate tumors, are potent antigen-presenting cells (APC) with the unique capacity to activate T cells, initiate specific primary immune response and support non-specific defense mechanisms. Activation or mobilization of the immune response by stress proteins released during the necrotic death of the cancer cells is in an agreement with the observation that the HPMA copolymer-bound drug, unlike free drug, induces necrosis [62–65].

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