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Biorecognizable Polymers: Design, Structure, and Bioactivity

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BIORECOGNIZABLE POLYMERS: DESIGN, STRUCTURE, AND BIOACTIVITY

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ABSTRACT

This paper provides a review of the relationship between the structure of biorecognizable N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers and their aggregation in solution with concomitant changes in biorecognition. To test the hypothesis that side-chain terminal moieties play a critical role in the association process, HPMA copolymers containing different stimuli sensitive hydrophobic moieties at side-chain termini (azobenzene, spiropyran, chlorin e_6, and phthalocyanine) were synthesized, and their solution and biological properties evaluated. The results obtained are of importance for the design of polymeric drug carriers as well as for the design of supramolecular systems with specialized functions.

INTRODUCTION

The synthesis, analysis, and characterization of biorecognizable polymers offers a great challenge to polymer science. Energetically favorable interactions of polymer conjugates with recognition systems developed during evolution call for a high level of complementarity of the receptor and ligand structures. The characterization of the structure-property relationship of biorecognizable polymers requires an interdisciplinary approach. In addition to a detailed analysis of polymer structure

and characterization of their properties by physicochemical methods, a repertoire of sophisticated biological assays has to be used to evaluate biological properties of the polymer conjugates. It is important to choose such biological characterization methods which permit the correlation to be made between the chemical and supramolecular structures of the conjugates and their biological response.

Supramolecular polymer chemistry, a highly interdisciplinary field of science, is emerging and receiving increased attention [1]. Several attempts have been made to demonstrate the relationship between the supramolecular structure of polymer systems and their recognition. The light induced change in the configuration of azobenzene groups in side-chains and/or crosslinks of macromolecules has been suggested as a tool for reversible optical storage [2], as a way to reverse antibody-antigen recognition [3], for photoregulation of enzyme activity [4], as well as for photocontrol of membrane permeabilities [5]. Recently, synthetic glycopolymers were synthesized and found to possess a much higher affinity with lectins than simple glycosides, based on "polymeric sugar-cluster effects" [6, 7]. A supramolecular assembly that transfers light energy absorbed by a self-assembled aggregate of zinc chlorin molecules to a single acceptor molecule has been prepared [8].

POLYMER DESIGN

During the last decade we have designed [9-11], developed [12, 13], and evaluated [14-23] targetable N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-anticancer drug conjugates. Phase I clinical data [24] seem to indicate their great potential in cancer therapy. The rationale of using water soluble polymeric drug carriers [25] is in the different mechanism of cell entry when compared to low molecular weight drugs. Attachment of drugs to macromolecular carriers changes their pharmacokinetics. While low molecular weight drugs can penetrate into all cell types by diffusion, their attachment to macromolecules limits cellular uptake of polymer-drug conjugates to the endocytic route. Internalized macromolecules are transferred via endosomes (receptosomes) into the lysosomal compartment of the cell [25]. The specificity of lysosomal enzymes can be used as the basis for controlled intracellular delivery [26-28]. Targeted polymer-drug conjugates can be prepared by attaching a ligand which is complementary to a receptor/antigen on the target cell thus raising the specificity of the delivery system to a higher level [11, 13].

The design of polymeric carriers of bioactive compounds in general, and of anticancer drugs in particular has to be based on a sound biological rationale.

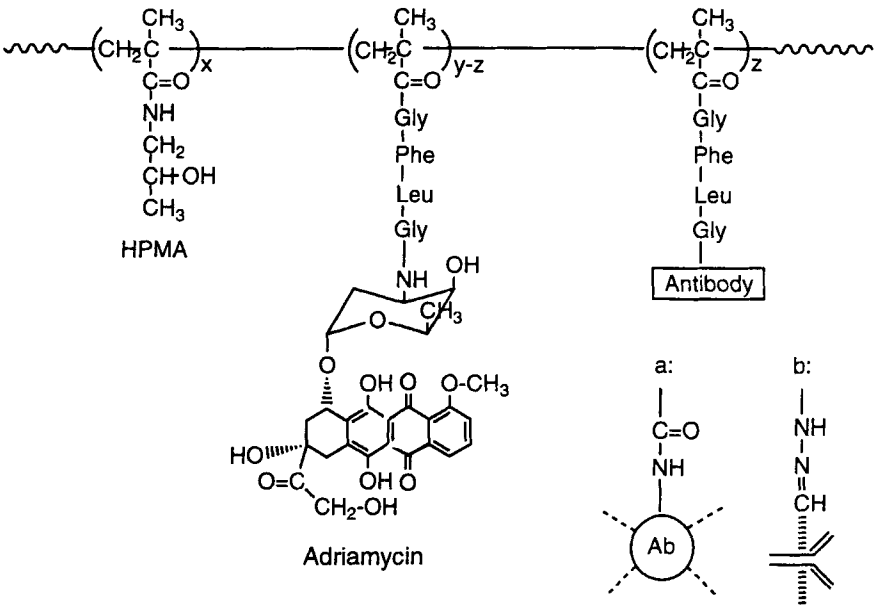

Figure 1. Structure of a targetable HPMA copolymer-anticancer drug (adriamycin) conjugate. Adriamycin (doxorubicin) is bound via a Gly-Phe-Leu-Gly sequence recognizable by lysosomal cysteine proteinase, cathepsin B. Monoclonal OV-TL16 antibody (targeting moiety) may be bound using different chemistries, e.g., (a) via ε-amino groups of lysine residues, or (b) via oxidized carbohydrate moieties [17].

HPMA copolymer-anticancer drug conjugates have to be designed in such a way that the covalent bond between the carrier and the drug is stable in the blood stream but susceptible to enzymatically catalyzed hydrolysis in the lysosomal compartment of the cell. To match the specificity of lysosomal thiol proteinases (cathepsin B) a hydrophobic oligopeptide sequence has to be used [27, 28]. The biological requirements result in a polymer conjugate structure containing a hydrophilic backbone and hydrophobic side-chains (Figure 1). It is well known that the solution properties of such amphiphilic copolymers will depend on many factors including the amount of side-chains (drug loading) per polymer chain.

To understand the relationship between the structure of amphiphilic polymers and their solution properties a series of model stimuli sensitive hydrophobic moieties were attached to side-chain termini of HPMA copolymers (Figure 2). Their solution properties and biorecognition were evaluated as described below.

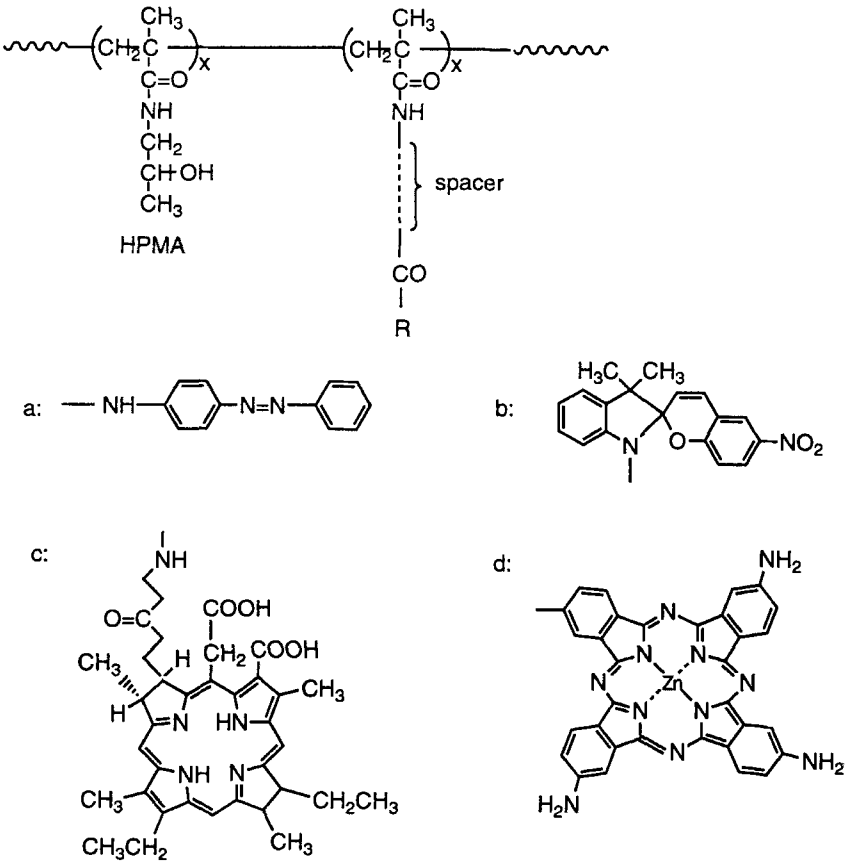

Figure 2. Structure of amphiphilic HPMA copolymers containing low molecular weight hydrophobic moieties (azobenzene (a), spiropyran (b), chlorin e_6 (c), phthalocyanine (d)) at side-chain termini.

ASSOCIATION OF AMPHIPHILIC POLYMERS IN SOLUTION

There have been few experimental investigations dealing with the association of water soluble copolymers containing low-molecular weight hydrophobic moieties at side-chain termini [29, 31-37]. Such copolymers form supramolecular clusters by random association of flexible macromolecules (Figure 3). The driving force for the association process is the attractive interaction between the hydrophobic moieties which results in the formation of intra- and/or inter-chain "point contacts" [33, 38] or more complicated multiplet-like hydrophobic domains [34]. The process is similar to the branching of macromolecules by covalent bonds. The only

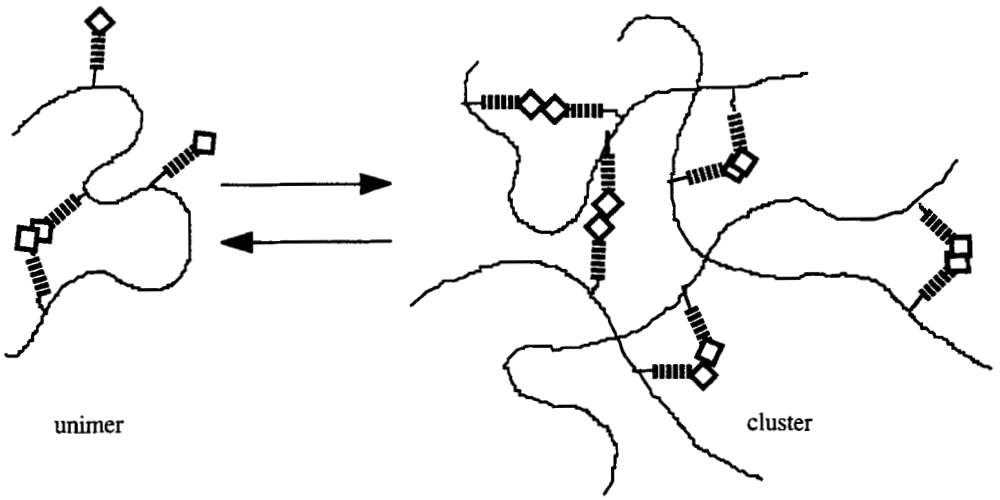

Figure 3. Random association of flexible random copolymers by "point contacts" of low-molecular weight hydrophobic moieties at side-chain termini.

difference being that the junctions between the polymers are not permanent. Instead, they open and close in dynamic equilibrium. The lifetime of the complexes is longer than the measurement time in typical static and dynamic light scattering experiments [39]. This kind of association is delicate compared to the hydrophobically associating water soluble polymers because only weak attractive forces act between the hydrophobic low molecular weight moieties. Therefore, this association process can be easily influenced by external stimuli [32]. In general, the dynamically associating structures of these polymers are typical for biorecognizable synthetic polymers, and polymeric drug delivery systems in particular. The clustering of polymeric drug carriers complicates their intercompartmental transport and their biorecognition by complementary structures at the cellular surface and/or in subcellular compartments, such as lysosomes [29].

Random Association of Macromolecules

Hydrophobic Interactions

Aggregation or self-assembly phenomena of hydrophilic polymers with low-molecular weight hydrophobic moieties at side-chain termini have been observed several times in aqueous solutions [29, 31-37]. We have previously shown that association phenomena occur in aqueous solutions of HPMA copolymers

TABLE 1. Solution Parameters of HPMA Copolymers Containing Gly-Leu-Phe-NAp Side-Chains Determined from Measurements of Integral and Quasi-Elastic Light Scattering: 25°C, TRIS Buffer pH 8 [Data from Reference 29].

Copolymer No.	x mol-%	M_w g mol^{-1}	R_h nm	Estimated assoc. number N
1	6.9	130 000	6.1	5
2	3.3	54 000	4.6	2
3	1.5	42 000	4.2	1

x mol.-% of modified side-chains,
M_w the total molar mass,
R_h the hydrodynamic radius of micelles.

containing p-nitroaniline attached to the copolymer by oligopeptide side-chains [29, 35]. These copolymers associate in water forming micelles having p-nitroaniline in the core and hydrophilic polymer forming the shell. Both, the association number and compactness of the micelles were found to depend on the content of hydrophobic side-chains. The results obtained [29] are shown in Table 1.

The hydrophobic moieties bound to side-chain termini of polymeric amphiphiles have a prominent role in the association process. Copolymers with amphiphilic properties in aqueous solutions seem to possess an advantage over other self-assembling systems in that the shape, size, and stability of the associates can be influenced relatively easily by changing external conditions which affect the solubility of the hydrophobic moieties.

In order to test the hypothesis that the side-chain terminal moieties play a critical role in the association process, an attempt was made to control copolymer association reversibly by photoirradiation. The objective was attained by incorporating a photochromic chromophore (azobenzene) into the side-chains of HPMA copolymers using 4-(acryloylamino)azobenzene as a comonomer [32]. The photochromic effect was associated with the *cis-trans* isomerization around the N=N double bonds. At room temperature, in the dark, the azo chromophores are expected to be primarily in the more stable *trans* configuration. Successive irradiation at different wavelengths produced reversible photoisomerization. The *trans* isomer is planar and practically apolar, whereas the *cis* configuration is not planar, bearing a high dipole moment, thus enhancing the copolymer's solubility.

TABLE 2. Effect of Irradiation ($\lambda \approx 360$ nm) on Parameters of HPMA Copolymer Containing Photochromic Azobenzene Side-Chains in Aqueous Solutions [Data from Reference 32].

Copolymer No.	x_a	Initial parameters M_w	R_h	N	Irradiation (1 h) M_w	R_h	N	Recovery (96 h) M_w	R_h	N
	mol-%	g mol^{-1}	nm		g mol^{-1}	nm		g mol^{-1}	nm	
4	4.4	6.4×10^4	6.4	1.1	6.2×10	6.4	1	-	-	-
5	8.3	1.0×10^5	6.0	1.2	8.7×10^4	5.4	1	9.0×10^4	5.4	1
6	9.3	3.2×10^7	97.0	311	2.1×10^5	12.2	2*	2.4×10^5	11.8	2.3
7	11.5	3.8×10^8	111	3350	4.0×10^7	92.5	3855	5.8×10^7	94.0	564
8	12.0	3.9×10^8	118	2980	1.8×10^8	106	1780	2.6×10^8	107	2470

*data after 3 hour irradiation

The solubility of HPMA copolymers in water was found to be a function of the molar content of azobenzene containing comonomer, x_a, attached to the HPMA backbone. While copolymers with $x_a = 4.4$ and 8.3 mol-%, respectively, were water soluble, forming macromolecular solutions (with a very low content of dimers), copolymers with $x_a \geq 9.3$ mol-% dissolved in water only as aggregates as the polymer concentration was slowly increased. The association parameters of copolymer samples dissolved in aqueous solutions are listed in Table 2. The molar mass, M_w, hydrodynamic radius, R_h, and association number, N, increase significantly at x_a (9.3 mol.-% indicating cluster formation in solution (Table 2).

The affect of irradiation by UV light ($\lambda \approx 350$ nm) on molecular and aggregate characteristics are shown in Table 2. While the dimensions of copolymer 4 having the lowest content of azobenzene ($x_a = 4.4$ mol-%) were practically insensitive to irradiation, a small decrease of M_w and R_h, relative to the initial values, was observed for copolymer 5 ($x_a = 8.3$ mol-%). This was due to the dissociation of dimers as a consequence of the enhanced solubility of the macromolecules with the photoinduced cis configuration of the azobenzene moieties. A pronounced effect of UV irradiation was observed in solutions of copolymer 6 which was close to its solubility limit. Large aggregates composed of 311 macromolecules dissociated upon irradiation to smaller ones with N = 2.

The thermal recovery process, i.e., the spontaneous conversion of the *cis* configuration to the more stable *trans* configuration, resulted only in a partial back-conversion of the hydrodynamic parameters. The most stable aggregates, those with the highest content of azobenzene (copolymer 8), showed a significant increase in M_w and N over the course of a four day recovery time; N changed by 39% to reach 83% of the initial value (Table 2), whereas practically negligible changes were found in the case of copolymer 6. Only a small collapse of unimer micelles due to a decrease in the water solubility of the *trans* configuration was observed. It appeared that more compact unimer and dimer micelles were preferred over swollen clusters because of their lower free energy.

It is difficult to imagine that the structure of clusters, particularly in the case of large clusters, would be similar to that of classic micelles with hydrophobic azobenzene oriented into the core and the hydrophilic backbone chains forming the shell of the spherical micelles. Since the solution properties of HPMA copolymers having azobenzene groups in their side-chains studied in aqueous solvents and ionomers in low-polarity solvents are very similar in many aspects, it is plausible to propose the same mechanism of aggregation in both cases, assuming point-like contacts [38] and/or multiplet-like formation [40]. Ringsdorf *et al.* indirectly observed multiplet-like structures in hydrophobically associating systems using fluorescent probes which were sensitive to the microviscosity and local polarity of hydrophilic regions [41]. Recently, the same laboratory revealed the existence of interpolymeric hydrophobic domains (multiplets) by experiments based on non-radiative energy transfer in mixtures of copolymers having side chains labeled either with naphthalene or pyrene probes [42].

We observed the formation of multimolecular clusters in aqueous solutions of N-(2-hydroxypropyl)methacrylamide copolymers containing side-chains terminated in Zn(II)-phthalocyanine (ZnP-HPMA copolymers) by light scattering and spectroscopic methods [30, 33]. Based on the results of spectroscopic measurements, it was determined that random association of ZnP-HPMA copolymers is primarily controlled by point contacts formed by Zn(II)-phthalocyanine (ZnP) dimers/or higher aggregates. These dimers are formed in aqueous solutions only. The water molecules play a specific role in binding together the ZnP molecules by hydrogen bonds. The association tendency of ZnP was increased by the addition of electrolytes and decreased by the addition of organic solvents or surfactants. We have used the differences in absorption spectra of ZnP monomers and dimers to estimate the level of ZnP dimerization in mixed solvents of TRIS buffer (pH=7.4) with DMSO. The effect of surfactants on cluster formation and ZnP dimerization

has also been studied. Thus, complete information about the clusters and their structure was obtained [30, 33].

In summary, copolymers containing hydrophobic side chain moieties that tend to form strong interactions, e.g., mesogenic azobenzene [32, 34] or Zn(II)-phthalocyanine [30, 33] seem to form large clusters by random association through point contacts and/or multiplet-like domains while weakly interacting hydrophobic moieties such as p-nitroaniline [29, 35] probably prefer to form small micelle-like clusters with hydrophobic moieties in the core and hydrophilic polymer chains forming the micellar shell. In order to resolve this problem, it will be necessary to analyze a variety of copolymers with different hydrophobic moieties bound to side chain termini.

Electrostatic Dipole-Dipole Interactions

Recently, copolymers of acrylamide having anionic, cationic and zwitterionic comonomers have been studied [43]. Unusual "antipolyelectrolyte behavior", as well as the formation of polymeric micelles, was observed in solutions of copolymers with zwitterionic groups in their side-chains. Intramolecular associations were indicated by drastic increases in the viscosity. We have studied the solubility and association of HPMA copolymers containing N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES) side-chains in aqueous solvents using light scattering methods [44]. The weight-average molar masses, diffusion coefficients and hydrodynamic sizes of the copolymer molecules and their clusters were studied as a function of HEPES content, solvent pH, and copolymer concentration. The copolymers having a high content of HEPES containing side-chains (60-100 mol%) associated in aqueous buffers, forming multi-chain aggregates (clusters). The formation of these clusters was strongly dependent on solvent pH. Well defined clusters were observed at pH values between 6 and 7 (the hydrodynamic radius, R_h, about 115 nm), while a broad distribution of larger clusters ($R_h \approx 250$ nm) was found at pH = 8 and 10. These clusters disappeared completely at pH = 3. The observed effect of solvent pH on the solution properties of copolymers is explained qualitatively by the electrolyte behavior of the HEPES molecules. The association found at solvent pH between 6 and 7 was related to the zwitterionic structure of HEPES containing side-chains which are formed in the vicinity of the half-neutralization point of the copolymers (pH $\approx$ 6.5). The attractive electrostatic forces among ion pairs (dipoles) of zwitterionic HEPES moieties appeared to be responsible for both the intermolecular and intramolecular associations. A direct analogy with the observed behavior of ionomers could be used for the interpretation of supramolecular structure and properties of these copolymers.

COVALENT ATTACHMENT VIA SIDE-CHAIN TERMINI

Photocrosslinking of proteins has been associated with cataractogenesis and with photohemolysis of red cells [45]. One suggested mechanism involves the photosensitized oxidation of histidine (tryptophan, or tyrosine) residues in one protein molecule followed by a nonphotochemical coupling with an amino group of another protein molecule to form covalent crosslinks [46]. We have shown that the mechanism of these reactions may be evaluated using tailor-made synthetic model macromolecules. HPMA copolymers containing ε-amino caproic acid side-chains terminating in histidine or lysine residues were used to model the photosensitized crosslinking reactions of proteins [47]. The extent of photocrosslinking, as sensitized by Rose bengal, was estimated by measuring the increase in the viscosity of model copolymer solutions after various periods of illumination. The extent of intermolecular crosslinking was estimated from the changes in molecular weight distribution of samples before and at the end of illumination as determined by size-exclusion chromatography. Photodynamic crosslinking occurred between P-Acap-His molecules and between P-Acap-His and P-Acap-Lys molecules. The higher the concentration of macromolecules in the solution, the higher the yield of intermolecular crosslinking. Oxygen was necessary for crosslinking, and azide inhibition studies indicated the involvement of singlet oxygen.

Several factors may influence the crosslinking reaction between two macromolecules. From the structural point of view, the number of attachment points and the concentrations of the macromolecules in solution are of decisive importance. Assuming the validity of the hypothesis that the crosslinking is mediated by singlet oxygen, factors such as the type and concentration of photosensitizer, concentration of oxygen, pH, light intensity, and temperature have been evaluated and their influence on the extent of the photocrosslinking reaction determined.

The conclusions from the influence of reaction conditions on the crosslinking of model HPMA copolymers were applied to the photosensitized crosslinking of RNase A [48]. The irradiation of solutions of RNase A in the presence of Rose bengal resulted in the progressive formation of dimers, trimers, and tetramers. Chemical modification of His residues (with diethyl pyrocarbonate) and/or Lys residues (with acetic acid N-hydroxysuccinimide ester) in the enzyme decreased crosslinking, suggesting the participation of these two amino acid residues in the reaction. Treatment of crosslinked RNase A and its His, Lys and Lys/His derivatives for 5 minutes at 97°C in a dithiothreitol/sodium dodecyl sulfate mixture efficiently ruptured a major part of the photodynamically formed crosslinks;

treatment with the detergent alone had no effect. Similar results were obtained with the crosslinked amino acid-containing HPMA copolymers, suggesting that photodynamic crosslinks involving His-His and His-Lys interactions are chemically the same in RNase A and the copolymer model. In summary, we have shown that photocrosslinking of proteins can be modelled using tailor-made synthetic copolymers. The results obtained contribute to the understanding of the mechanism of processes which take place during photodynamic cancer therapy [49].

BIORECOGNITION AND BIOACTIVITY

We have shown previously that the solution properties of HPMA copolymer - anticancer drug conjugates influence their biorecognition. The rate of enzymatically catalyzed release of drugs (drug models) bound to side-chain termini [29] as well as the quantum yield of singlet oxygen formation [21, 30] by photosensitizer - HPMA copolymer conjugates are influenced by the formation of aggregates in solution. The changes in the binding affinity of OV-TL16 conjugates and their homogeneity (in terms of binding affinity) are a consequence of conformational changes in the antibody structure. A series of physicochemical methods were employed to characterize the HPMA copolymer - anticancer drug - antibody conjugates and to correlate their physicochemical and biological properties [17].

HPMA copolymers containing p-nitroaniline attached to the copolymer via oligopeptide side-chains [29] associate in water forming micelles having p-nitroaniline in the core and the hydrophilic polymer forming the shell of the micelle. The recognition of side-chains by α-chymotrypsin was impaired for copolymers 1 and 2 in Table 1 [29]. The higher the degree of aggregation, the lower the biorecognition with a concomitant decrease in the rate of release of the chromogenic p-nitroaniline group.

HPMA copolymers containing chlorin e_6 at side-chain termini were studied using dynamic light scattering, spectroscopic, fluorescence quenching, and time-resolved fluorescence decay techniques. Intramolecular aggregation of chlorin e_6 species with concomitant decrease in the quantum yield of oxygen uptake was detected. The amount of aggregation was dependent on the composition of the copolymer, on pH, and on the presence of detergent molecules [50].

HPMA copolymers containing side-chains terminated in Zn(II) 4,9,16,23-tetraaminophthalocyanine aggregated in aqueous solutions [30, 33] as detected by UV and fluorescence spectra, and QELS measurements. The dimerization of phthalocyanine moieties resulted in decreased quantum yields of oxygen uptake during the photooxidation of furfuryl alcohol [30]. Similar data were obtained with

HPMA copolymer - chlorin e_6 conjugates [21]. Aggregation of oligopeptide side-chains terminated in chlorin e_6 resulted in decreased recognition by cathepsin B (lysosomal cysteine proteinase) with a concomitant decrease in the quantum yield of singlet oxygen formation.

Recently, we have studied the interactions of amino sugar containing HPMA copolymers with Tetragonolobus purpureas (Lotus tetragonolobus) lectin immobilized on 4% beaded agarose by frontal affinity chromatography [51]. HPMA copolymers containing side-chains terminated in N-acylated aminosugars, namely, fucosylamine, galactosamine, glucosamine and mannosamine were synthesized and their recognition evaluated. As expected, fucosylamine containing HPMA copolymers showed specific binding to the lectin. The binding (dissociation) constant (K_d) for HPMA copolymers was determined. It was found that K_d depends on the amount of fucosylamine residues per molecule. An increase in fucosylamine content from 9.9 to 33.2 mol-% resulted in a decrease of K_d by one order of magnitude. The incorporation of hydrophobic side-chains into the copolymers increased the non-specific binding to the lectin. The photoinduced isomerization of bound spiropyranes to merocyanines was accompanied by changes in the macromolecular conformation and consequently influenced the binding of the HPMA copolymers to the lectin. The results obtained demonstrate the impact of the structure and molecular conformation of sugar containing HPMA copolymers on their molecular recognizability.

CONCLUSIONS

HPMA copolymers containing various low molecular weight hydrophobic moieties at side-chain termini were synthesized and evaluated. The aim of the research was to clarify the role of molecular architecture in the formation and recognition of supramolecular structures in solution. The influence of structure, charge, polarity and spacing from the HPMA copolymer backbone on aggregation and recognition was determined. It was demonstrated that copolymers containing photochromic groups (e.g., azobenzene, spiropyran) at side-chain termini change their supramolecular structure upon external stimuli. The control of the association process by external stimuli could open new fields of applications of these aggregates, e.g., in pharmaceutical and environmental sciences.

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