Immobilization of Aminothiols on Poly(oxyalkylene phosphates). Formation of Poly(oxyethylene phosphates)/Cysteamine Complexes and Their Radioprotective Efficiency

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The necessity to apply near-toxic amounts of radioprotective drugs to achieve adequate protection during radiation treatments represents a major problem in human medicine. One of the promising strategies to suppress the toxicity of these drugs involves their incorporation into biocompatible polymers. In this study cysteamine (Cy) was attached to poly(oxyethylene phosphate), POEP, via an ionic bond. Radioprotection of *E. coli* B cells by this substance and its acute toxicity on male C57 BL mice were measured. The toxicity of Cy immobilized within the poly(oxyethylene phosphate) was significantly lower in comparison to pure Cy while its radioprotective efficiency remained high at half the maximum tolerable dose. The high radioprotective efficiency of the Cy/POEP complexes was further confirmed on mice at different polymer molecular weight characteristics, drug immobilization degrees, application times, and doses. It was found that POEP with molecular weight 4700 Da and containing 24% repeating units with attached Cy has the highest protection potential combined with a depot effect.

Introduction

Human and technological factors continue to contribute toward the necessity for efficient protection against nuclear radiation. Cysteamine (Cy) hydrochloride is a well-known conventional chemical radioprotector. Bacq reported the first clinical applications of this substance several decades ago.[1] Cy was administered in single or multiple doses before or immediately after irradiation, and it protected against the symptoms of radiation sickness. Its protective effect was manifested mainly by the more rapid recovery after the termination of the therapy of treated patients.[2,3] The need to use near-toxic amounts of radioprotective drugs to achieve adequate protection, however, still represents a major problem in human medicine.[4] It is well-known that polymer−drug conjugates could improve drug localization in the target tissue, diminish drug exposure in potential sites of toxicity, and optimize drug release rate.[5] If Cy could be immobilized on biodegradable polymers, it would be possible to reduce its toxicity while preserving its good radioprotective capability. Poly(ethylene glycol), PEG, is a hydrophilic synthetic polymer, which has been extensively studied as biocompatible polymer drug carrier.[6] The fact that the polymer backbone is not biodegradable in vivo is one of the few potential shortcomings of PEG. The other major disadvantage is the low drug-loading capacity of this polymer, limited by the availability of only two attachment sites at the termini of the linear PEG molecule. Promising candidates for an immobilization template are the poly(oxyalkylene phosphates), POAP, a family of biodegradable, hydrophilic, and nontoxic polymers that contain PEG moieties and suitable multifunctional sites for immobilization.[7] They possess several favorable features: (i) excellent solubility in aqueous media; (ii) existence of multiple anchoring positions that would extend their drug-loading capacity (P−OCH_3 and P═O groups in every repeating unit); (iii) broader molecular weight range of administration because after hydrolysis the individual segments (low molecular weight PEG) will be safely and efficiently excreted.

The basic strategy for the synthesis of radioprotective substances involves a 'molecule combination' based on covalent or ionic bonds between the basic components.[8,9] Other alternatives are the absorption complexes where the formation is caused by donor−acceptor interactions and hydrogen bonds.[10]

The major goals of the present study are the immobilization of the conventional chemical protective agent cysteamine (Cy) on POAP via ionic bonds and the evaluation of the radioprotective efficiency of the complexes formed. These experiments combine and extend our line of inquiry toward development of low-toxicity polymers for drug encapsulation and transport[11] and capable of binding highly efficient radiomodifying agents.[7g]

Chemistry. Synthesis of Poly(oxyethylene phosphonate), 1. 1 was synthesized via transesterification of dimethyl hydrogen phosphonates by poly(ethylene

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Scheme 1

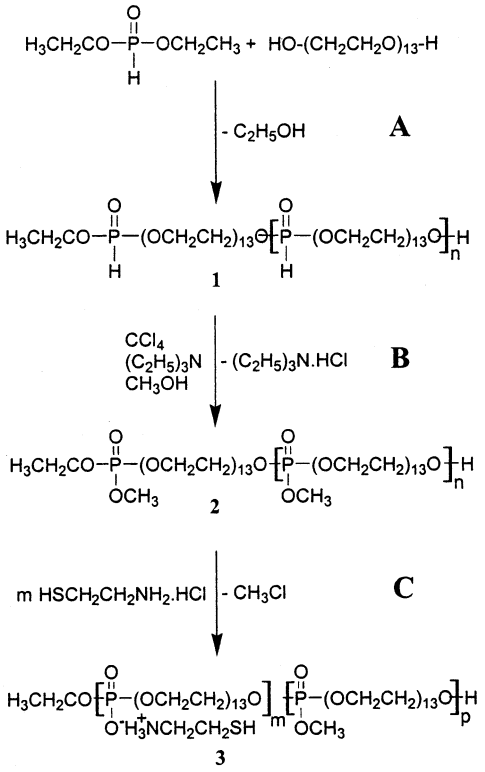

Table 1. Molecular Weights and Chemical Compositions of Poly(oxyethylene phosphates) and Their Cy Complexes

PEG M_n	POEP (**1**) M_n	POEP−Cy (**3**) m^a
200	1900	**3a**: 0.99
400	3000	**3b**: 0.99
600	4700	**3c**: 0.99
600	4700	**3d**: 0.49
600	4700	**3e**: 0.24

[a] m is the degree of Cy immobilization and is calculated from the integral intensities of the P-atoms in POEP−Cy (Scheme 3, **3**) − phosphate structure (^{31}P{H} NMR spectrum, $\delta = 1.33$ ppm): diphosphate anion (^{31}P{H} NMR spectrum, $\delta = 0.32$ ppm).

glycol)s, PEGs, with different average molecular weights (200, 400, and 600 Da) in a molar ratio 1:1, Scheme 1A. The structure of **1** was elucidated by a combination of ^{1}H, ^{31}P, and ^{13}C NMR spectroscopy. The polymeric character of the transesterification products was confirmed by size-exclusion chromatography (SEC). The number average molecular weight (M_n) of **1** was between 1900 and 4700 Da depending on the initial PEG used, Table 1. The average degree of polymerization (DP) could be also estimated by ^{31}P NMR from the ratio of phosphorus atoms at the end-groups to the phosphorus atoms in the repeating units. It is found that for **1** DP = 6−7. SEC measurements showed that the molecular weight distribution of all polymers investigated was rather narrow ($M_w/M_n = 1.15-1.2$).

Synthesis of Poly(oxyethylene phosphate), 2. The Atherton−Todd reaction is used to transform **1** into poly(oxyethylene phosphate), POEP, **2**, Scheme 1B. The structure of **2** is confirmed by the analysis of its NMR spectra. The absence of any resonance with large $^1J_{(P,H)}$ coupling constants of 700 Hz (characteristic of PH protons) could be regarded as a direct proof for the complete conversion of **1**.

Table 2. Influence of the Irradiation Dose on the Cell Survival Rate (N/N_0) for *E. coli B*

irradiation dose (Gy)	N/N_0 (%)
50	55.0000
100	1.2500
200	0.7250
400	0.0175
600	0.0028

Immobilization of Cy Hydrochloride on 2. The presence of methoxy groups in the poly(oxyethylene phosphate) predetermines two possible pathways for the immobilization through an ionic bond of amine-containing biologically active substances: (a) alkylation reaction; (b) dealkylation reaction. Both of them are affected by the reactivity of the α-carbon atom of the alkoxy group attached to the phosphorus (electrophilic center). In this work we performed the Cy immobilization by the dealkylation reaction, Scheme 1C. The results from the NMR spectroscopy indicate that Cy is attached to the POEP by an ionic bond (**3** in Scheme 1C). The chlorine elemental analysis shows that the content of the free cysteamine hydrochloride in products **3** is less than 1%. The degree of Cy incorporation along the polymer chain could be conveniently regulated by the initial amounts used in the final stage of immobilization and is measured by ^{31}P{H} NMR spectroscopy, (Scheme 1 and Table 1).

Biological Results. Acute Toxicity. The acute toxicities of the newly synthesized compounds **3** and their constituents are evaluated in vivo (intraperitoneal administration in mice).

POEP toxicity is tested for doses starting at 500 mg/kg and increasing in arithmetic progression up to 2000 mg/kg. It should be noted that in all cases investigated the administered polymer is nontoxic. Higher doses are not tested because they are beyond the practical application range of radioprotection amounts.

The investigation of the acute Cy toxicity yields the following data: maximum tolerable dose (LD_0) = 167 mg/kg; LD_{50} = 265 mg/kg, as calculated by Probit analysis;[12] and absolutely lethal dose (LD_{100}) = 377 mg/kg. The LD_{50} value found in this study is close to the previously reported results.[13]

The toxicity of POEP−Cy complexes is investigated at three distinct concentrations that would match the Cy toxic doses: 1037 mg/kg POEP−Cy (167 mg/kg Cy); 1645 mg/kg POEP−Cy (265 mg/kg Cy) and 2340 mg/kg POEP−Cy (377 mg/kg Cy). The effect is monitored up to 48 h after the intraperitoneal injection. It should be emphasized that for this time span the mice survival rate is 100% even at the highest dose applied. It could be assumed that the immobilization of Cy on POEP leads to a significant reduction of its toxicity.

Radioprotective Efficiency. The POEP−Cy radioprotective efficiency of **3c** is initially evaluated on *E. coli* B cells, a test frequently used for the determination of Dose Reduction Factor (DRF).[14] The strain survival after γ-irradiation in the interval from 50 to 600 Gy is estimated by the ability of the cells to form colonies—the ratio of the number of colonies in the irradiated probe (N) to the number of colonies in the nonirradiated sample (N_0). The results are presented in Table 2.

Table 3. Radioprotective Efficiency of Cy and POEP−Cy (**3c**) Evaluated by Dose Reduction Factor (DRF)

compound	dose[a]	lg *a*	lg *b*	*p*-value[b]	D_{37} [c]	DRF[d]
control		4.60	−0.08		55.7	
Cy	1/2	4.48	−0.07	0.77	58.7	1.05
POEP−Cy	1/2	5.48	−0.05	0.01	111.2	2.00
POEP−Cy	1/4	4.68	−0.06	0.04	74.3	1.33
POEP−Cy	1/8	4.87	−0.06	0.01	73.8	1.33

[a] The dose is expressed as a part of LD_0 for Cy. This value is 312 μg/mL and represents the maximal nontoxic Cy concentration whereby the growth is equal in the control and the treated tube. [b] Statistical probability factor. [c] The irradiation dose for 37% cell survival rate. [d] The values were found by division to the respective D_{37} with the control D_{37}.

The inactivation of the survival as a function of the dose can be described by the following analytical equation:

$$\log y = \log a + \log b \times D$$

where $\log y = \log N/N_0$, a and b are constants, D is the irradiation dose. The graphical solution of the equation enables the determination of D_{37} (dose for 37% cell survival)[15] that is used later in the investigation. The radioprotective efficiency of Cy before and after the immobilization on **2** (molecular weight 4700 Da) is determined by the dose reduction factor (DRF), defined as the ratio of D_{37} of the probe treated with radioprotectors to D_{37} of the control probe. The results are presented in Table 3. It is seen that the protective effect is well pronounced at high doses (1/2 LD_0), but it is also statistically significant at the minimal dose.

The protective effect of the complex POEP−Cy is then studied on mice after acute whole body gamma irradiation with 8 Gy ($LD_{70/30}$: 70% dead rate after 30 days). The preparation is administered in doses equal to 1/8−1/2 mmol/kg Cy immobilized on the polymer carrier (1/16 to 1/4 of the maximum tolerable dose of Cy). It would be interesting to evaluate the influence of the polymer molecular weight on the protective effect of the formulation. For this purpose POEP−Cy complexes with different molecular weight, but containing the same amount of Cy (1/2 mmol Cy/kg corresponding to 1/4 of maximum tolerable dose) are tested, Table 4. Comparative experiments with single application of pure polymer carriers with molecular weights ranging from 1900 to 4700 show that POEP itself has no radioprotective effect.

The calculated values for PF and PI reveal that the immobilization of cysteamine on the poly(oxyethylene phosphate)s results in well expressed increase of their radioprotection efficiency. The complex with molecular weight 3000 Da shows the highest protective effect. Substances **3a** and **3c** could also be described as "very effective" (++++ protective index), according to the classification of the protective agents.[4]

The correlation between the amount of the Cy immobilized on the polymer-carrier and the radioprotective effect of the composition is investigated using three preparations with different degree of Cy immobilization (**3c**: $m = 0.99$, **3d**: $m = 0.49$ and **3e**: $m = 0.24$, Scheme 1C, Table 1). The results are presented in Table 5. The degree of the immobilization (m) of the cysteamine onto the poly (oxyethylene phosphate)s has a pronounced influence on the radioprotective efficiency of the preparations. Substance **3e** with degree of the immobilization 24% ($m = 0.24$) protects 100% of the irradiated animals and the therapeutic width of the preparation (RF = 2.00; PI = 19.33) is also better. It should be emphasized that the Cy dose applied therewith is 8 times less than the optimal one used in radioprotection.

It is often expected that polymer−drug carriers with suitable molecular weight characteristics and chemical composition would have extended times of body circulation leading to prolonged drug action. The time efficiency of POEP−Cy complexes is investigated with the experimental animals being exposed to radiation 24 h after the drug application, Table 6. A slightly expressed depot effect is observed with all complexes, the effect being clearly visible again with **3e** ($M_n = 4700$ Da; $m = 0.24$, Table 6). This cysteamine complex shows a depot protective effect at Cy concentration representing only 1/16 of the maximum tolerated dose (PF = 1.70; PI > 65.78). The observed phenomena (Tables 5 and 6) could not be explained only by the polymer character of the POEP−Cy complex. It is obvious that the increase in the degree of polymerization is improving the protective efficiency of the formulations and their depot effect due most probably to their increased drug loading and prolonged circulation. The degree of cysteamine immobilization (m), however, affects the properties and pharmacological behavior more profoundly. The seemingly surprising higher efficiency observed at lower drug loading ($m = 0.24$) could be possibly explained by the higher stability of these complexes. The introduction of numerous charged sites along the polymer chain (see Scheme 1C, **3**) would certainly affect the hydrolyzability of the POEP−Cy adducts. Thus, higher Cy contents (and lower degrees of polymerization) would favor faster hydrolysis and consequently more rapid excretion.

Conclusions

The immobilization of the clinically tested chemical radioprotector cysteamine on poly(oxyethylene phosphate) leads to the formation of a complex between the polymer carrier and Cy. Experimental mammals (mice) are used to study the toxicological characteristics of the new polymer derivative. A significant reduction of Cy toxicity is observed after the immobilization on POEP. No toxic effects appear even after the administration of the absolute Cy lethal dose. The results obtained after radiation experiments with *E. coli* show the statistically significant radioprotection efficiency of the poly(oxyethylene phosphate)/Cy complex. The maximum DRF value attained is 1.996. The effect is distinctly expressed in all three doses investigated (1/2, 1/4, and 1/8 of Cy LD_0) while pure Cy did not show any protective action even after the administration at 1/2 of the maximum tolerable dose. Experimental animals (mice) were used to study the acute toxicity and radioprotective effect of cysteamine immobilized on poly(oxyethylene phosphate)s with different molecular weight characteristics and chemical compositions. The molecular weight of the polymer carrier and the degree of cysteamine immobilization play a key role in the radioprotective mechanism of action of the polymer−drug complexes.

Experimental Section

Dimethyl phosphonate (Fluka AG) was purified by standard procedures. PEG with average molecular weight 200, 400, and 600 Da (Fluka AG) was dried prior to use at 120 °C by bubbling

Table 4. Correlation between POEP Molecular Weights and Radioprotective Efficiencies Their Cy Complexes

substance	POEP M_n	POEP m	dose[a] (POEP−Cy mg/kg)	dose[a] (mmol Cy/kg)	30-day survival[b] (%)	PF[c]	PI[d]
3a	1900	0.99	195	1/2	60	1.6	>15.5
3b	3000	0.99	278	1/2	90	1.9	>18.4
3c	4700	0.99	390	1/2	70	1.7	>16.4
Cy	78		39[e]	1/2	30	−	−
control	−		−	−	30	−	−

[a] The applied amount of Cy with each polymer preparation corresponds to 1/4 of its maximum tolerable dose (167 mg/kg). [b] Survival of animals after 8 Gy irradiation ($LD_{70/30}$). [c] PF = protection factor calculated as 1 + fraction of survivors; PF values range is between 1 (0% survival) and 2 (100% survival). [d] PI = protection index of POEP−Cy calculated as LD_{50} (265 mg/kg)/lowest effective dose (mg/kg). [e] Amount of pure Cy.

Table 5. Influence of the Cy Degree of Immobilization on the Protective Effect of the Polymer−Drug Complex

substance	m	dose[a] (mg POEP−Cy/kg)	dose[a] (mmol Cy/kg)	30-day survival[b] (%)	PF[c]	PI[d]
3e	0.24	1560	1/2	100	2.00	>19.33
3d	0.49	780	1/2	50	1.50	>14.50
3c	0.99	390	1/2	70	1.70	>16.44
Cy	−	39	1/2	30	−	−
control		−	−	30	−	−

[a] The applied amount of Cy with each polymer preparation corresponds to 1/4 of its maximum tolerated dose (167 mg/kg). [b] Survival of animals after 8 Gy whole body irradiation ($LD_{70/30}$). [c] PF = protection factor calculated as 1 + fraction of survivors; PF values range is between 1 (0% survival) and 2 (100% survival). [d] PI = protection index of POEP−Cy calculated as LD_{50} (265 mg/kg)/lowest effective dose (mg/kg).

Table 6. Time Dependence of the POEP−Cy Radioprotection Efficiency (depot effect)

substance	M_n	m	dose[a] (mg POEP−Cy/kg)	dose[a] (mmol Cy/kg)	30-day survival[b] (%)	PF[c]	PI[d]
3a	1900	0.99	195	1/2	40	−	−
3b	3000	0.99	278	1/2	40	−	−
3c	4700	0.99	390	1/2	50	1.50	>14.50
3d	4700	0.49	185	1/4	40	−	−
3e	4700	0.24	93	1/8	70	1.70	>65.73
Cy	113.61		39	1/2	10	−	−
control	−		−	−	30	−	−

[a] The applied amount of Cy with each polymer preparation corresponds to 1/4 of its maximum tolerated dose (167 mg/kg). [b] Survival of animals after 8 Gy whole body irradiation ($LD_{70/30}$). [c] PF = protection factor calculated as 1 + fraction of survivors; PF values range is between 1 (0% survival) and 2 (100% survival). [d] PI = protection index of POEP−Cy calculated as LD_{50} (265 mg/kg)/lowest effective dose (mg/kg).

a stream of dry argon through the melt and simultaneous application of dynamic vacuum.[7e] Cy hydrochloride was purchased from Fluka AG and used as supplied. Carbon tetrachloride and dichloromethane were dried over P_2O_5 and distilled immediately before use.

All NMR spectra (^1H, ^{13}C, and ^{31}P) were recorded on a Bruker 500 MHz instrument in $CDCl_3$ solutions. Hydroxyl numbers were determined by the acetylation method on a Mettler apparatus. SEC measurements were performed on a Waters 244 line equipped with four Ultrastyragel columns with pore sizes 100, 100, 500, and 500 Å and tetrahydrofuran as the carrier solvent. The molecular weights were calculated using a conventional calibration with PEG standards.

Poly(oxyethylene phosphonate), 1. A typical procedure is described as follows: PEG 600 (11.2 g, 0.018 mol) and diethyl phosphonate (2.63 g, 0.019 mol) were placed in a three-necked flask equipped with a magnetic stirrer, thermometer, and condenser. The process was carried out at 150 °C. The progress of the reaction was monitored by the amount of ethanol evolved. When this amount reached 90% of the theoretical value, the temperature was increased to 165 °C, and the system was placed under dynamic vacuum (1 mmHg). The polytransesterification was completed in 6 h. **1** was obtained as a waxy solid in 94% yield. ^1H NMR ($CDCl_3$): δ (ppm) 1.36 (t, $^3J_{(H,H)}$ = 7.1 Hz, C*H*$_3$CH$_2$OP), 3.48−3.61 (m, OC*H*$_2$CH$_2$), 4.11−4.14 (m, POC*H*$_2$CH$_3$), 6.79 (d, $^1J_{(P,H)}$ = 701.0 Hz, P*H* end group), 6.80 (d, $^1J_{(P,H)}$ = 715.1 Hz, P*H* repeating unit); $^{13}C\{H\}$ NMR ($CDCl_3$): δ 14.9 (d, $^3J_{(P,C)}$ = 4.8 Hz, -*C*H$_3$), 60.8 (d, $^2J_{(P,C)}$ = 2.5 Hz, POCH$_2$), 63.8 (*C*H$_2$OCH$_2$); ^{31}P NMR ($CDCl_3$): δ 7.71 (d of q, $^1J_{(P,H)}$ = 702.5 Hz, $^3J_{(H,H)}$ = 9.38 Hz, *P* end group), 9.98 (d of q, $^1J_{(P,H)}$ = 711.8 Hz, $^3J_{(H,H)}$ = 9.28 Hz, *P* repeating unit).

Poly(oxyethylene phosphate), 2. A typical experiment is presented. Dichloromethane (9 mL), carbon tetrachloride (22.5 mL), triethylamine (0.94 g), and methanol (0.72 mL) were placed in a three-necked flask equipped with a magnetic stirrer, thermometer, reflux condenser, and a dropping funnel. A solution of **1** (2.3 g, 0.0036 mol of repeating units) in dichloromethane (13.5 mL) was added dropwise at ambient temperature under continuous stirring. The reaction was allowed to proceed for 24 h. After filtration of the precipitated triethylamine hydrochloride, the filtrate was concentrated and the polymer product (**2**) was precipitated by addition of diethyl ether. **2** was purified by dissolution in *N*,*N*-dimethylformamide and reprecipitation in diethyl ether. The isolated product was dried at 30−40 °C under reduced pressure (1 mmHg). Yield 2.6 g (100%). ^1H NMR ($CDCl_3$): δ (ppm) 1.36 (t, $^4J_{(H,H)}$ = 7.1 Hz, C*H*$_3$CH$_2$OP), 3.48−3.61 (m, −OC*H*$_2$CH$_2$-), 3.76 (d, $^3J_{(P,H)}$ = 11.6 Hz, −POC*H*$_3$), $^{13}C\{H\}$ NMR ($CDCl_3$): δ 14.9 (d, $^3J_{(P,C)}$ = 4.8 Hz, *C*H$_3$), 54.6 (d, $^2J_{(P,C)}$ = 5.6 Hz, POCH$_3$), 60.8 (d, $^2J_{(P,C)}$ = 2.5 Hz, POCH$_2$), 63.8 (*C*H$_2$OCH$_2$); ^{31}P NMR ($CDCl_3$): δ 0.57, 1.28 (octet, $^3J_{(P,H)}$ = 10.05 Hz, $^3J_{(H,H)}$ = 8.14 Hz, *P*OCH$_3$ + *P*OCH$_2$CH$_3$).

Immobilization of Cy Hydrochloride on 2. Poly(oxyethylene phosphate) (5.2 g, 7.7 mmol of repeating units) and Cy hydrochloride (0.85 g, 7.7 mmol) were mixed in a two-necked flask fitted with a magnetic stirrer, reflux condenser, and a thermometer. The reaction was carried out at 110 °C and was stopped after the evolution of methyl chloride ceased.

The crude product **3** was dissolved in chloroform and precipitated by addition of diethyl ether. **3** was dried under dynamic vacuum at 30−40 °C. Yield 5.8 g (95.4%). ^{1}H NMR ($CDCl_3$): δ (ppm) 1.36 (t, $^3J_{(H,H)}$ = 7.1 Hz, CH_3CH_2OP), 3.48−3.61 (m, OCH_2CH_2), 3.76 (d, $^3J_{(P,H)}$ = 11.6 Hz, $POCH_3$), 4.11−4.14 (m, $POCH_2CH_3$), 4.65 (s, S*H*), 8.19 (s, NH_3); ^{13}C{H} NMR ($CDCl_3$): δ 14.9 (d, $^3J_{(P,C)}$ = 4.8 Hz, CH_3), 39.3 (CH_2SH), 43.1 (NCH_2), 54.6 (d, $^2J_{(P,C)}$ = 5.6 Hz, $POCH_3$), 60.8 (d, $^2J_{(P,C)}$ = 2.5 Hz, $POCH_2$), 63.8 (CH_2OCH_2); ^{31}P NMR ($CDCl_3$): δ 0.32 (PO^-), 1.34 (octet, $^3J_{(P,H)}$ = 10.05 Hz, $^3J_{(H,H)}$ = 8.14 Hz, $POCH_3$ + $POCH_2CH_3$).

Bacteria and Animals. The bacterial strain used in the study was *E. coli B* (Cat. # 417, National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria, 1998). The experimental animals were male mice with body weight 20−22 g (C_{57}BL line).

Radioprotective Evaluation. A semisolid bovine agar (0.7%) was inoculated with the *E. coli B* strain and after growth the cells were fixed in sterile paraffin. The morphology of bacterial cells was analyzed microscopically. The density of the bacterial suspension was standardized at $3 \times 10^8 - 5 \times 10^8$ cells/mL.

Acute Toxicity. The toxicity was determined after a single intraperitoneal administration of the preparation with arithmetic progression in groups of five animals. The substances were dissolved in physiological solution with pH = 7.2 ex tempore and applied via single 0.5 mL intraperitoneal injections. The death rate was registered in 1, 24, and 48 h intervals after application of the corresponding substance.

Irradiation and Registration of the Protection Efficiency. The investigated protectors were added to aliquots of the bacterial suspension (1 mL/tube) 15 min before irradiation, and the resulting mixtures were irradiated with an exposing dose in air of 50−600 Gy at room temperature together with the control probes. The irradiation was performed by ^{60}Co source at a dose rate of 1.2 Gy/s. After the treatment the cells were plated on Petri dishes (100 mm/dm). For each dose of irradiation dilutions (10×) in a physiological solution were prepared and 0.05 mL was placed on the Petri dishes. The latter were cultivated at 37 °C. The results were evaluated after 20 h by the colony-forming ability of the cultures as measured by an automatic electronic counter.

Pure Cy and POEP−Cy complexes underwent radiobiological screening. Mice were divided into groups of 10 animals each. They received 8 Gy whole body irradiation (source ^{137}Cs; dose rate − 0.021Gy/s; $LD_{70/30}$). Preparations were administered 15−20 min before the radiation exposure. In experiments studying the depot effect the preparations were applied 24 h before irradiation.

Irradiated animals were maintained in standard facilities. Control and treated groups of animals were checked daily over 30 post irradiation days to record deaths. Radioprotective characteristics were assessed using an integral indicator − animal survival rate and biometrics as protection factor (PF) and protective index (PI).[14] The coefficient PF reflects the probability of the organism to be protected. PF is between 1 (no survival) and 2 (100% survival) The PI has the advantage to give the degree of protection as well as the therapeutic width.

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