THE USE OF COUMARIN DERIVATIVES IN THE PREPARATION OF FLUORESCENCE-LABELLED POLY[N-(2-HYDROXYPROPYL)METHACRYLAMIDE]

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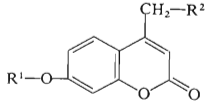
The possibilities of preparation of fluorescence-labelled poly[N-(2-hydroxypropyl)methacrylamide] by using 7-substituted 2*H*-1-benzopyran-2-ones (coumarins) in radical processes were investigated.

The interest taken in the fluorescence labelling of polymers for the investigation[1] of physical properties or for analytical purposes has been constantly increasing in recent years. In our preceding study[2] we reported the preparation of some novel 7-substituted coumarins (2*H*-1-benzopyran-2-ones), suitable for uses as fluorescence labels. The objective pursued by us in this study has been an investigation of the prospects of preparation of a fluorescence-labelled poly[N(2-hydroxypropyl)methacrylamide][3,4] by using coumarin derivatives with a view of the utilization of the resulting copolymers in the living organism.



I, $R^1 = H$, $R^2 = NHCO(CH_3)C{=}CH_2$
II, $R^1 = CH_2{=}C(CH_3)CO$, $R^2 = H$
III, $R^1 = CH_2{=}C(CH_3)CO$, $R^2 = COOH$
IV, $R^1 = H$, $R^2 = COOH$
V, $R^1 = CH_3CO$, $R^2 = COOH$

EXPERIMENTAL

Infrared spectra (KBr disc technique) were recorded with a Zeiss UR 20 spectrometer. UV spectra were recorded with a Cary 14 apparatus. Molecular masses, M_w, were determined from the light scattering dilute solutions of samples in dimethylformamide (measurements using a Photo-

goniodiffusometer Fica); the respective refractive index increment values were inter- and extrapolated from the dependence of the refractive index increment on the composition of the analyzed copolymers. GPC operations were performed in a GPC apparatus built at the Institure (dimethylformamide, column packing Porasil EDP). Fluorescence spectra were measured using an apparatus also built at the Institute with an automatic correction of the recorded spectra.

The preparation of 4-methacryloylaminomethyl-7-hydroxy-2*H*-1-benzopyran-2-one (*I*), 7-methacryloyloxy-4-methyl-2*H*-1-benzopyran-2-one (*II*), 7-methacryloyloxy-2-oxo-2*H*-1-benzopyran-4-acetic acid (*III*), 7-hydroxy-2-oxo-2*H*-1-benzopyran-4-acetic acid (*IV*) and 7-acetoxy-2-oxo-2*H*-1-benzopyran-4-acetic acid (*V*) has been described earlier[2].

Homogeneous Radical Solution Copolymerization of N-(2-Hydroxypropyl)methacrylamide with *I*, *II*, and *III*

N-(2-Hydroxypropyl)methacrylamide (further called monomer) and freshly recrystallized comonomers *I*, *II*, *III* were dissolved in dimethylformamide (0·87M of monomer and 0·62M of comonomer). According to the selected initial ratio of both copolymerization components, corresponding amounts of the individual solutions were placed into a 20 ml polymerization ampoule, and made up to the total volume 15 ml by dimethylformamide. The molar concentration of the initiator, 2,2′-azobis(isobutyronitrile) was $3 \cdot 10^{-3}$ mol l^{-1} and the resulting overall monomer concentration was 0·578M in all cases. The contents of the ampoule were vigorously flushed with nitrogen; the ampoules were then sealed and left in a bath at 60°C for 30 h. The contents of the ampoules were precipitated into a twentyfold excess of acetone and dried to constant weight *in vacuo* over P_2O_5.

Heterogeneous Radical Solution Polymerization of N-(2-Hydroxypropyl)methacrylamide in the Presence of *IV* and *V*

The monomer and freshly recrystallized components *IV* and *V* were dissolved in acetone (1·25M of monomer and 0·2M of the other component). The following procedure was the same as in the preceding case, the molar concentration of initiator being $3{\cdot}28 \cdot 10^{-2}$ mol l^{-1}. After vigorous stirring the ampoules were sealed and left at 50°C for 24 h; the contents of the ampoules were filtered, thoroughly washed with acetone and dried to constant weight *in vacuo* over P_2O_5.

Purification of Polymers

The polymers were purified by repeated reprecipitation and the GPC method or by dialysis. The latter was carried out with dialysis tubing manufactured by SERVA (Visking Dialysis Tubing 8/32). The polymers were dialyzed until the dialysate did not exhibit a constant intensity of fluorescence for five days, after which the solutions were lyophilized.

The gels were statistically extracted for three weeks with dimethylformamide (the solvent over the gel was exchanged every other day), transferred into acetone, and dried.

Analysis of Polymers

The polymer composition was calculated from UV spectra (dimethylformamide). The calculation was based on the molar extinction coefficients of individual compounds ($\varepsilon^{I}_{322} = 1{\cdot}39 \cdot 10^4$, $\varepsilon^{II}_{312} = 0{\cdot}94 \cdot 10^4$, $\varepsilon^{III}_{312} = 0{\cdot}94 \cdot 10^4$, $\varepsilon^{IV}_{327} = 1{\cdot}43 \cdot 10^4$, $\varepsilon^{V}_{312} = 0{\cdot}85 \cdot 10^4$). In the case of microgels, the value used was the difference between extinction at the maximum of absorption of the

given monomer and the absorption value at 450 nm. The accuracy of the method in repeated measurements of the same sample was 2%.

RESULTS AND DISCUSSION

The fluorescence labelling of the polymer of N-(2-hydroxypropyl)methacrylamide (further called monomer) was performed by the homogeneous solution copolymerization of the monomer with coumarin derivatives *I*, *II*, *III* as labels. Table I shows that comonomer *I* enters into the copolymer more slowly than the monomer. At low contents of *I* this label meets the requirements on fluorescent labels. With increasing content of *I* the molecular mass of copolymers increases; at still higher contents of the label, gel formation sets in.

To elucidate these processes, the monomer was copolymerized with *II* and *III* under the same conditions as with *I*. While the content of comonomer *II* in the resulting copolymer is lower than that of *I*, the content of comonomer *III* is roughly comparable. However, completely soluble copolymers can in these cases be obtained only at low contents of comonomer in the mixture, *i.e.*, with comonomer *II* up to 15% and with *III* only up to 5%. Higher contents of both comonomers in the initial polymerization mixture (25–80% for *II*, 15–25% for *III*) lead to the formation of microgels or even of gels. If unambiguously soluble copolymers are obtained, the weight conversion of the copolymer is virtually the same as in the copolymerization of the monomer with *I*. Comonomers *II* and *III* are of course not suited for fluorescence labelling, because the intensity of their fluorescence is low.

TABLE I

Homogeneous Solution Copolymerization of N-(2-Hydroxypropyl)methacrylamide with 4-Methacryloylaminomethyl-7-hydroxy-2*H*-1-benzopyran-2-one (*I*) in Dimethylformamide

Polymerization temperature 60°C, polymerization time 30 h. Concentrations given in mol %.

Composition of mixture		Conversion wt.%	Copolymer composition		$\bar{M}_w$
[*I*]	[monomer]		[*I*]	[monomer]	
100	0	87·8	—	—	gel
80·00	20·00	82·5	—	—	gel
50·0	50·0	88·5	42·3	57·7	370 000
25·0	75·0	80·6	23·5	76·5	110 000
15·0	85·0	81·2	13·8	86·2	96 000
5·0	95·0	78·0	4·4	95·6	93 000
2·0	98·0	82·1	1·8	98·2	73 000
0	100	86·4	—	—	55 000

Gels arising by the solution homopolymerization of *II* or *III* are identical, as documented by IR spectra. This is obviously due to the decarboxylation reaction observed with 4-carboxymethyl derivatives of coumarins[2]. In order to evaluate the extent to which the transfer reaction participates in gel formation, the same polymerizations were carried out in the presence of tetrabromomethane. With *II*, only low-molecular weight compounds are formed at elevated concentrations of the transfer agent (2–5 mol %), while with *III* soluble polymers are obtained; gels are formed at concentrations of the transfer agent below 0·5 mol %. The influence of the transfer agent on the crosslinking process may be regarded as sufficiently supporting the radical transfer hypothesis. It may be assumed that the critical domain in the molecule responsible for the transfer is the methylene group in position 4; gel formation in the polymerization of *II* is moreover corroborated by the decarboxylation reaction.

It may be concluded, therefore, that compounds containing the carboxymethylene group in position 4 on the coumarin skeleton play a certain important role in radical processes. For this reason, an attempt has been made to initiate the polymerization of the basic monomer by decomposition products of acids *IV* and *V*. It appears, however, that only a major amount of fluorochrome in the reaction mixture leads to the formation of the polymer, at a very low conversion (about 10%). Some 2% of the polymer are also formed in the absence of fluorochrome, which however is due to the known tendency of the monomer to spontaneous polymerization. An attempt to polymerize vinyl acetate by using *IV* and *V* has failed. All these findings indicate that 4-carboxymethyl derivatives cannot be employed as initiators, in spite of their participation in radical processes. We tried to corroborate this assumption by the solution polymerization of the basic monomer in the presence of *IV* and *V* with 2,2'-azobis(isobutyronitrile) as initiator (heterogeneous system). It was found that the presence of coumarins in the initial reaction mixture affects insignificantly the molecular mass of the resulting polymer and the magnitude of conversion. Fluorescence measurements show that in the purified product the fluorochrome

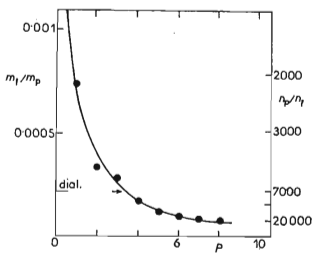


FIG. 1

Dependence of the Amount of 7-Hydroxy-2-oxo-2*H*-1-benzopyran-4-acetic Acid Adsorbed on Poly[N-(2-hydroxypropyl)methacrylamide] on the Number of Reprecipitations (*P*)

m Mass (g), *n* number of mol, *f* index for 7-hydroxy-4-coumarinacetic acid, *p* index for polymer.

content is the higher, the higher its content was in the original polymerization mixture; under comparable conditions the content of fluorochrome *V* is always higher (3 to 7 times). If the fluorochrome content in the initial mixture increases fifty times, the amount in the product increases approximately five times for *IV* and seven times for *V*.

It seems appropriate here to characterize the efficiency of purifying operations. The GPC method (Potasil EDP, dimethylformamide) does not yield products with the fluorescence background so low as to make possible their utilization in the final repurification. This is suggested by a correlation between the number of reprecipitations and the amount of adsorbed fluorochrome on poly N-(2-hydroxypropyl)methacrylamide in the blank test, *i.e.* after mechanical mixing of the same weight parts of the polymer and fluorochrome *IV* in dimethylformamide and after precipitation into acetone (Fig. 1). It cannot be therefore argued with certitude that in polymerizations of the monomer in the presence of selected fluorochromes the only bond formed between the reaction components is the chemical bond. We believe, however, that most of the fluorochromes are bound chemically, as may be inferred from the following experiment and speculation:

If one deacylates a chosen sample of the reaction product of the monomer with comonomer *V*, which is bound in the product approximately three times better than comonomer *IV*, the obtained polymer contains approximately the same amount of fluorochrome as before this operation, *i.e.* about three times higher than according to Fig. 1 corresponds to a similarly purified polymer in which fluorochrome *IV* is bound only physically.

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