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Direct Condensation Reaction For Grafting of Poly (Ethylene Glycol) Methyl Ether on Poly (Methacrylic Acid-co-methyl Methacrylate)

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Abstract: *Synthesis and characterization of copolymers based on poly(ethylene glycol) mono methyl ether-g-poly(methacrylic acid-co-methyl methacrylate) via a polymeric precursor method is reported. Grafting was accomplished based on direct condensation reaction in the presence of dicyclohexylcarbodiimide as an esterification promoting agent catalyzed by dimethylamino pyridine. PEG-grafted copolymers were characterized using different spectroscopic techniques and their biocompatibility was also studied. Appearance of bands assigned to the ester functional groups in Fourier transform infrared spectra was used for structure characterization of the grafted copolymer. Grafting efficiency was assured the performance of grafting reaction. Cytotoxicity evaluation of the grafted copolymer using L929 fibroblast cell line elucidated acceptable biocompatibility profile also applicability of the copolymers for biomedical applications.*

Keywords: *Graft copolymer, Methoxy Poly(ethylene glycol), poly(methacrylic acid-co-methyl methacrylate), Direct polycondensation, Esterification promoting agent.*

1. INTRODUCTION

Looking for novel materials with favorable new properties have been an everlasting field in material sciences. This is especially the case with the polymeric ones upon their multifarious possible variations in the nature (or ratio) of the initial monomer(s), synthesis way, catalysts, final processing *etc.* which in turn will end to a new material hence properties[1-9]. Finally obtained properties such as mechanical[1], thermal[2] or biological[3-6] ones have in turn an ultimately deterministic effect on its final feasible application. During the last decades more attention has been focused on the modification of the currently available materials to yield new properties without need to huge costs and inevitably long time required to develop a new material from test tube to market[5-9].

For this purpose, two major categories of physical and/or chemical approaches are being adopted in modification of polymers for biomedical applications. For instance,

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plasma surface modification of Polyamide 6 is frequently reported to increase its wettability[10-14] or to improve cell adhesion and proliferation on poly(L-lactide) and poly(D,L-lactide-*co*-glycolide)[15]. Chemical treatments of biomaterials are also widely reported in a number of different methods from grafting[16-18] to crosslinking[19-21], and structural modifications like hydrogenation[22], halogenations[23] and hydrohalogenation[24, 25].

Polyethylene glycol (PEG) with its desirable and intrigue properties such as biocompatibility, bioresorbability up to 20 kD (nominal molecular weight), ionic conductivity, wettability and resistance to protein adsorption[26] has been found an outstanding role in modification of biological entities, polymers and surfaces[26-30]. Modification of acrylic resins using PEG has attracted much attention [29-42]. Generally, these amphiphilic PEG-grafted copolymers were prepared by either (co)polymerization of vinyl-derivatives of PEG[32-36] or synthesis of polymeric precursors followed by PEG conjugation via polymeric analogous reactions[37-42]. A procedure using polymeric precursors was adopted by Chiu *et al.* to prepare a polymeric drug carrier via synthesis of a linear acrylic resin from methyl acrylate, stearyl methacrylate, acrylic acid and PEG acrylate and subsequent reaction with mPEG in different molar ratios and mPEG nominal molecular weights in benzene[37]. Where in the last decade of ex-century synthesis and characterization of a variety of PEG-grafted copolymers upon polymeric precursors method was reported by many researchers such as Wesslen and Wesslen[38], Derand and Wesslen[39], Twaik *et al.*[40], Thierry *et al.*[41] and Jannasch *et al.*[42] however; many aspects have not been fully covered by their research including direct esterification of carboxyl and hydroxyl functional groups of polyacids and mPEG; respectively also evaluation of their biocompatibility and cell supporting capacity.

In this study, mPEG was grafted on these MA-*co*-MMA copolymers via a polymeric precursor method which is not previously reported for this copolymer. This method was based on adoption of a direct condensation reaction in the presence of dicyclohexylcarbodiimide (DCC) as an esterification promoting agent catalyzed by (dimethylamino)pyridine (DMAP) which is not previously reported. PEG-grafted copolymers were characterized using Fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance spectroscopy (^{1}HNMR) techniques. Grafting efficiency and their biocompatibility was also evaluated.

2. MATERIALS AND METHODS

2.1. Materials

MA-*co*-MMA was a kind gift from Röhm Pharma GmbH (Darmstadt, Germany) which dried by leaving in a forced-air convection oven at 110 °C for 24 hrs. before use. Monomethoxy poly(ethylene glycol) (mPEG) of 350 & 750 g.mole^{-1} nominal molecular weights were supplied by Fluka (Ronkonkoma, USA) and dried using azeotropic distillation in toluene. *N,N'*-Dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino) pyridine (DMAP) were purchased from Merck chemicals (Darmstadt, Germany) and used without further purification. Acetone (Merck Chemicals Co., Darmstadt, Germany) was purified by distillation under ambient

pressure before heating under reflux condition with successive quantities of potassium permanganate. It was then dried using anhydrous potassium carbonate, filtered from the desiccant and stored over type 4A molecular sieves. Toluene and other solvents and reagents were all of analytical grade and purchased from Merck and used as received.

Acid value of MA-*co*-MMA copolymer was determined according to United States Pharmacopoeia (USP)[43]. Briefly, a precise weight of copolymer was dissolved in acetone (1% w/v) then titrated with NaOH solution (0.1 N) in the presence of phenol phthalein as an indicator until its color changing was remained constant for at least 15 seconds. Each mL of the used titrant is equal to 0.0937 mole of methacrylic acid. Methacrylic acid percentage in the backbone was determined using the following equation:

$$Methacrylic\ Acid(\%) = \frac{NaOH(mL) \times 0.8609}{MA\text{ - }co\text{ - }MMA(g)} \times 100 \quad (1)$$

2.2. Grafting Reaction

In a typical procedure, pre-purified and dried MA-*co*-MMA copolymer was dissolved in 100 mL of neutralized acetone and charged into a 250 mL three-necked reaction flask equipped with reflux condenser, dropping funnel and thermometer. DMAP (0.1% mol/mol to mPEG) was added to this mixture and heated to 45 °C under reflux condition. A respective amount of mPEG was dissolved in 50 mL of acetone to yield 10, 20 or 30% molar ratio to methacrylic acid units. DCC (1% mol/mol to mPEG) was added to the mPEG solution at room temperature. This solution was added drop-wise to the reaction flask during 15 minutes. The precise compositions used in the synthesis of grafted polymers are shown in Table 1.

The reaction mixture was then left overnight under reflux condition afterwards transferred to a refrigerator at 5 °C for 24 hrs. Needle-like dicyclohexylurea crystals were removed from the reaction media by filtration. Water was used as a non-solvent to the solution and the product was removed by decantation. The same purification process was repeated for 2-3 times and the resulting polymer was dried in a forced-air

Table 1
Feed Ratio Composition for Reactants used in the Synthesis of Grafted Copolymers

	MA-co-MMA	*mPEG*		*DCC*	*DMAP*
Sample Code	*(mole)*	*Mw(g.mole^{-1})*	*Mole*	*(mole)*	*(mole)*
10P350	0.0163	350	0.0016	0.0016	0.0001
20P350	0.0163	350	0.0033	0.0033	0.0003
30P350	0.0163	350	0.0049	0.0049	0.0005
10P750	0.0163	750	0.0016	0.0016	0.0001
20P750	0.0163	750	0.0033	0.0033	0.0003
30P750	0.0163	750	0.0049	0.0049	0.0005

convection oven at 70 °C for 12 hrs. The product appeared as a white, brittle and acetone-soluble powder which was collected from filter paper and stored at -5 °C in a desiccator until further use.

2.3. Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared (FTIR) spectra (4000-400 cm^{-1}) were acquired using an Equinox 55 spectrophotometer (Bruker, Germany) at 4 cm^{-1} resolution and 32 scans at room temperature. FTIR spectra of mPEGs were collected applying the materials on KBr disks while they were in liquid or molten state. Grafted copolymers were mixed thoroughly in 1:70 ratios with KBr in a mortar and pestle and equal weights (212 mg) were used to prepare compressed disks. All measurements were made in transmittance mode.

2.4. Proton Nuclear Magnetic Resonance Spectroscopy (^{1}HNMR)

Proton nuclear magnetic resonance spectra (^{1}HNMR) were recorded using a Bruker UltraShield 400 system (Bruker, Germany) at 25 °C to characterize the copolymer structure and confirm the inclusion of mPEG chains on MA-*co*-MMA backbone. Samples were dissolved in deuterated acetone and chemical shifts were recorded in ppm from the signal of tetramethylsilane.

2.5. Grafting Efficiency

Grafting efficiency (GE%) was evaluated by determination of unreacted carboxylic acid functional groups by a titration technique as previously described in the section under determination of methacrylic acid content of the copolymer. Titrations were performed kinetically on 0.5, 1, 2 and 4 hrs. after initiation of the grafting reaction *i.e.* complete addition of mPEG to the reaction flask. GE (%) was calculated according to the following equation:

$$GE(\%) = \frac{MA_I - MA_G}{MA_I} \times 100 \quad (2)$$

2.6. In Vitro Cell Culture

The mouse L929 fibroblast cell line (NCBI C-161; National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) were cultured in RPMI-1640 (GIBCO, Scotland) supplemented with 10% fetal calf serum (FCS) (Seromed, Germany), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma, Milwaukee, USA). L929 cells were then harvested with 0.25% trypsin-EDTA solution (Sigma, Milwaukee, USA) in phosphate-buffered saline (PBS, pH 7.4) and seeded onto the 96-well microtiter plates (NUNC, Denmark) at a density of 1×10^4 cells/well for direct contact tests. Tissue culture polystyrene (TCPS) was used as a reference. The cells were incubated at 37 °C in humified air with 5% CO_2.

2.7. Statistical Analysis

Statistical analysis was performed using MiniTab software (Release 11.12, Minitab Inc., State College, PA, USA). Data were reported as mean±standard deviation at significance level of $p < 0.05$. Differences were considered statistically significant when the p value was < 0.05.

3. RESULTS AND DISCUSSION

Using the previously described method in determination of acid value for MA-co-MMA copolymer; 28.02% methacrylic acid units (equivalent to 0.001625 moles per gram of dry polymer weight) was present in the polymer composition. Three levels of mPEG concentration in feed were considered for grafting i.e. 10, 20 and 30% (molar ratio to methacrylic acid units) to provide different copolymers regarding hydrophilicity. The effect of varying molecular weight of hydrophilic units on the final copolymer properties is also shown by Hashemi doulabi -et al.[44]. Grafted copolymers were synthesized by adopting a direct condensation procedure based on using an esterification promoting agent i.e. DCC. According to the supposed reaction mechanism for Steglich esterification i.e. the production of the ester bonds in presence of DCC and DMAP[45] the carboxylic acid functional groups present in MA-co--MMA copolymer will be converted to an O-acylisourea intermediate upon reaction with DCC, which offers reactivity similar to the corresponding carboxylic acid anhydride. The reactive intermediate then will form an acyl pyridinium species with DMAP (I). This will be followed by equilibration of (I) with the mPEG to produce ion pair (II). Grafted copolymer and stable dicyclohexylurea (DHU) will be generated via nucleophilic attack by R´O- on the acyl group of (II) when DMAP will also be recovered. By the way, a terpolymer of methyl methacrylate, methacrylic acid and monomethyl ethylene glycol methacrylate was synthesized via direct condensation reaction. The reaction mechanism is depicted in Figure 1.

FTIR spectra of starting MA-*co*-MMA copolymer along with the corresponding grafted material are depicted in Figure 2.

According to FTIR spectra of MA-*co*--MMA copolymer, specific signals related to hydroxyl (3443 cm^{-1}), methylene (2999 and 2953 cm^{-1}), carbonyl (1732 cm^{-1}) and alkyl-substituted ether (1155 cm^{-1}) functional groups were declined in the finally grafted material's spectra; whereas performance of the esterification reaction was guaranteed by the appearance of new methoxy and methyl ether band signals at 2853 and 1020 cm^{-1}, respectively. In Table 2 assignments for important signals of the initial copolymer and grafted materials were tabulated.

Proton nuclear magnetic resonance (^{1}HNMR) was used to guarantee FTIR results. Appearance of mPEG ethoxy protons signal at 1.2 ppm chemical shift clearly indicated the promotion of esterification reaction between MA-*co*-MMA and mPEG. Assignment of signals appeared in ^{1}HNMR spectra of starting materials and mPEG-grafted copolymer are as follows:

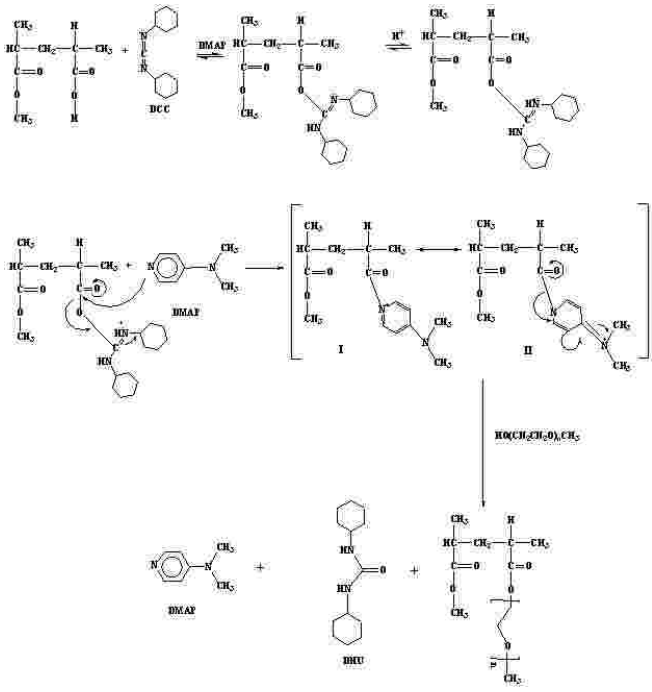

Figure 1: The Proposed Scheme for Grafting Reaction

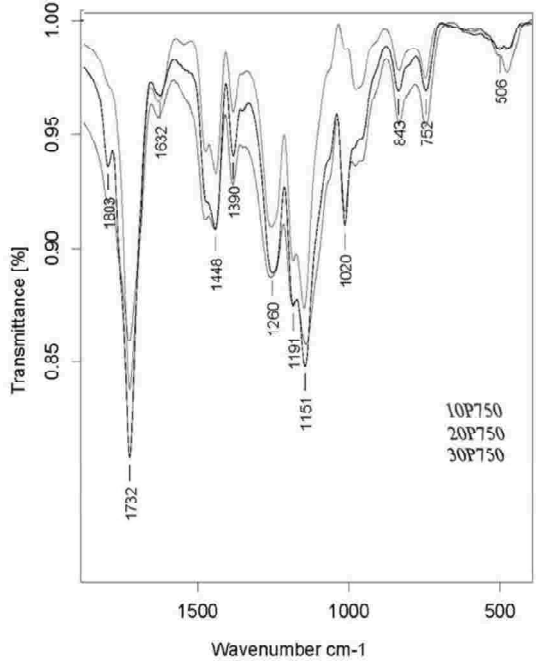

Figure 2: FTIR Spectra of PEG-grafted MA-*co*-MMA

Table 2
FTIR Spectral Data of Virgin MA-*co*-MMA and the Grafted Copolymer[a]

Wavelength (cm^{-1})		
MA-co-MMA	*Grafted Copolymer*	*Functional Group*
3443 b	3437 b	O-H (stretching)
2953 m	2951 m	C-H (asymmetric stretching)
	2853 m	O-CH_3 (stretching)
1733 s	1732 s	O=C-O
	1632 w	N-H (bending)
1447 m	1448 m	CH_2 (bending)
1390 m	1390 m	C-H (symmetric bend)
1265 s	1260 s	C-O
1191 s	1191 s	O=C-O-C
1155 s	1151 s	alkyl-substituted ether, C-O (stretching)
	1020 m	C-O (stretching)

[a]All values for wave number are in cm^{-1}. s: strong; b: broad; m: medium; w: weak.

^{1}HNMR: δ3.58 (methoxy protons of MMA), δ2.80 (methylene functional group of mPEG adjacent to the formed carboxyl ester), δ2.25 (methyl functional group of MMA repeating units), δ2.09 (CH_3 of deuterated acetone solvent signal), δ1.87 (methylene protons in mPEG backbone), δ1.28 (methylene protons on MA-*co*-MMA backbone), δ0.88 (methyl protons of MA-*co*-MMA side chains). The ratio of integrals for signal appeared at 2.8 ppm to any signal originated by copolymer backbone can be correlated to the degree of grafting. The ^{1}HNMR spectra of MA-*co*-MMA and mPEG-grafted copolymers are depicted in Figure 3.

Grafting efficiency (GE) was measured to assure the performance of grafting reaction. GE was increased upon increasing in mPEG percentage in the feed for a constant molecular weight as it is shown in Figure 4. This can be attributed to the

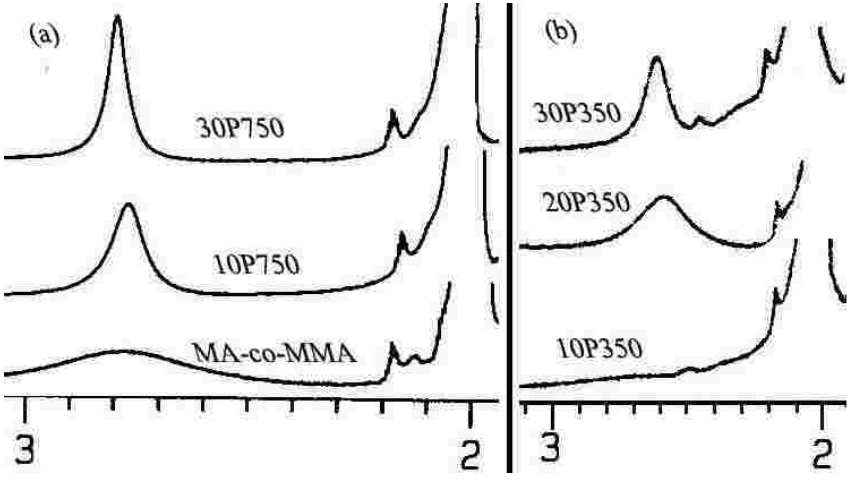

Figure 3: ^{1}HNMR Spectra of MA-*co*-MMA along with the Corresponding Grafted Materials

well-known mass effect law; however, GE was reduced upon increasing in the molecular weight of mPEG in the feed which can be attributed to the lower reactivity ratios of hydroxyl functional groups for higher molecular weight analogues of mPEG. The observed reduction in the GE upon increasing molecular weights of mPEG may also be assigned to the increasing potential of intra-molecular hydrogen bond formation between polyacid and polyol *i.e.* mPEG chains. The reaction time plays an important role in a condensation reaction which was evident in the final fortune of GE in long times when the same values were obtained for different molecular weights of mPEG especially in the case of higher feed ratios of mPEG.

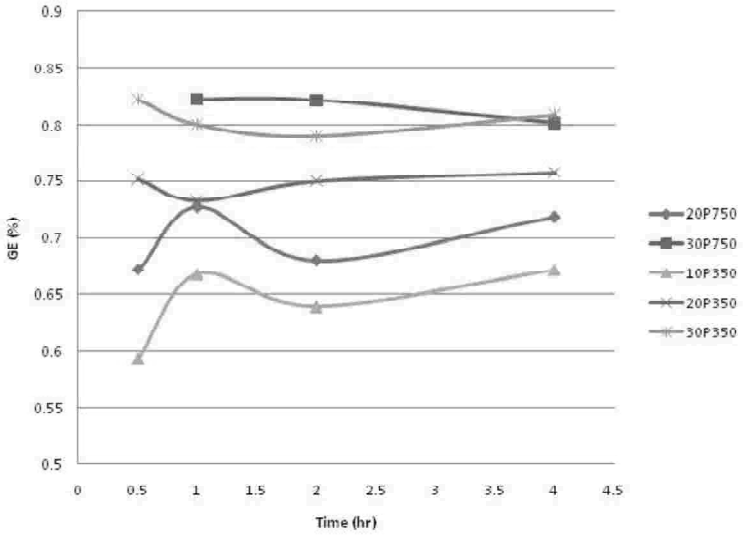

Figure 4: Grafting Efficiency of the Reaction for Different Compositions versus Reaction Time

Cytotoxicity evaluation of the grafted copolymers using L929 fibroblast cell line elucidated acceptable biocompatibility profile also applicability of the copolymers. Direct observation of cells in close proximity of the PEG-grafted powder revealed that a considerable amount of cells on the PEG-grafted copolymers started spreading and obtaining their fibroblastic morphology. The cell morphology of L929 after one week spreading is shown in Figure 5.

Exposed surface areas of the cells were obtained via image analysis using Image-pro plus software. Data were collected for at least 6000 cells in each sample and the test was run in quadricate. As it is evident from data represented in Table 3, cells were got more flattened upon introduction of mPEG into the structure of copolymer ($p<0.05$) but there were no statistically significant difference between 10, 20 and 30% mPEG grafted samples ($p>0.5$). According to the results significant changes will occur by incorporation a limited amount of mPEG in the structure of the grafted copolymer (10% mPEG) but incorporation of higher ratios of hydrophilic moieties will not end to better results.

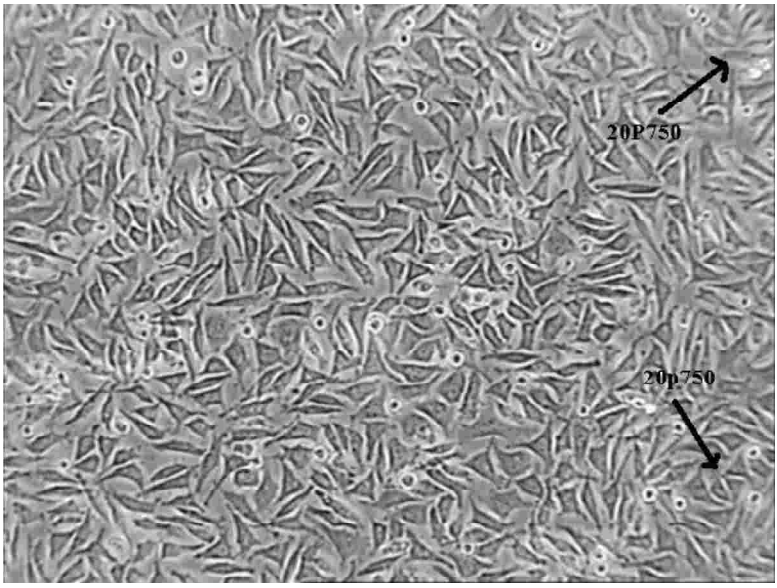

Figure 5: Cell Morphology of L929 Fibroblasts after Seven Days Culturing on 30P750

Table 3
Surface Area of L929 Fibroblasts Cultured on PEG Grafted Copolymer Samples after 1-week Exposure

Sample	*Cell Area (pixel)*
TCPS	15.10±5.06
10P750	42.99±19.26
20P750	56.57±11.52
30P750	69.84±48.88

4. CONCLUSION

A terpolymer of methyl methacrylate, methacrylic acid and monomethyl ethylene glycol methacrylate was synthesized by direct condensation reaction of mPEG and MA-*co*-MMA via Steglich mechanism using DCC as an esterification promoting agent in the presence of DMAP. Chemical structure of the resulting materials were characterized spectroscopically by FTIR and ^{1}HNMR techniques which clearly showed significant changes including the appearance of carbonyl ester signal band absorption in FTIR also chemical shift at 2.8 ppm in ^{1}HNMR spectroscopy which can be assigned to methylene groups of mPEG just adjacent to carbonyl functional groups of MA upon the reaction. GE was dependent on the initial mPEG molecular weights and its concentration in the feed ratio which was relieved in long reaction times. Moreover, seven days *in vitro* cytotoxicity examination to evaluate cellular proliferation in the neighborhood of grafted copolymer was in good agreement with the controls showing

higher biocompatibility profiles for samples grafted with 10% of mPEG in comparison to neat ones. Increasing mPEG content to higher values did not improve the results further on.

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Note

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