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Conformation and orientation of end-grafted (co)polyglutamates and (co)polyaspartates

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The conformation and orientation of end-grafted (co)polyglutamates and (co)polyaspartates were investigated using several infra-red spectroscopy techniques. The grafted polymers took on the same conformations as the corresponding free polymers; however, the change in conformation at higher temperatures did not take place. The orientation of the polymers grafted onto flat surfaces turned out to be bent towards the surface, but became more perpendicular after interdiffusion of *trans*-ethyl-β-apo-8'-carotenoate.

(Keywords: grafting; polyglutamates; polyaspartates)

INTRODUCTION

Most polyglutamates and polyaspartates are polypeptides with a stable secondary structure, and many theoretical investigations have been carried out to calculate the stability of these structures. Ooi *et al.*[1] have made calculations of the stability of some polypeptides, including poly(γ-methyl L-glutamate) (PMLG) and poly(β-methyl L-aspartate) (PMLA). It turned out that non-bonded interactions favour the right-handed α-helix in both polymers. However, in polyaspartate the interaction of the dipole of the side-chain ester with the dipole of the backbone amide group is more repulsive for the right-handed than for the left-handed helix. This causes a destabilization of the right-handed helix in PMLA, while in PMLG both the non-bonded and the dipole–dipole interactions favour the right-handed α-helix. Side-chain conformations also have a large effect on the conformation of the helical polypeptides. Both theoretical[2] and experimental[3–6] studies have shown that the conformation of a polyglutamate backbone can change with changing side-chain conformation. For many polyglutamates the most stable conformation is the right-handed α-helix, which is stabilized by intramolecular H bonding. The α-helical structure of the polyglutamates is the cause of some interesting properties. Poly(γ-benzyl L-glutamate) (PBLG) shows lyotropic liquid-crystalline behaviour in some solvents[7–10], while (co)polyglutamates with longer aliphatic side chains can form thermotropic liquid-crystalline phases[11–18].

Though polyaspartates tend to exist in the α-helical conformation too, their secondary structure is less stable than that of polyglutamates. Changing the size of the side chains or the polymer composition of (co)polyaspartates has a large effect on the secondary structure of these polypeptides[19–23], which can be in a right-handed (α_R) or left-handed (α_L) α-helix, or in a left-handed ω-helix (ω_L) in which the structure is stabilized by H bonding between the amide N–H bond and the side-chain ester. By heating the polymer, this structure can be changed irreversibly into a β-sheet conformation, which is stabilized by intermolecular H-bonds as well[22]. Upon dissolving the polypeptides in a hydrogen-bond-breaking solvent like dichloroacetic acid (DCA) or trifluoroacetic acid (TFA), the polymers turn into a random-coil conformation. The secondary structures of polypeptides can be determined experimentally with n.m.r. spectroscopy[24,25], circular dichroism[21,26–31], optical rotatory dispersion[26–28,32,33], X-ray diffraction[19,34] and *FT*i.r. spectroscopy[35–43].

This present research deals with the conformation and orientation of (co)polyglutamates and (co)polyaspartates, grafted onto microparticulate silica and glass and silicon slides, as derived from infra-red spectroscopy. The positions of the amide bands in the infra-red spectra of the polyglutamates and polyaspartates are characteristic of the conformation of the backbone, thereby allowing the determination of the molecular conformation of the grafted polymers (*Table 1*). In the α-helices the carbonyl bond of the amides is oriented parallel to the helix axis, and therefore the orientation of grafted helices can be determined with *FT*i.r spectroscopy by studying the dichroism of the carbonyl stretching band, using transmission and reflection spectroscopic techniques. With the polarization of the infra-red light parallel (p) to the plane of incidence, the absorbance ratio of the ester and the amide I carbonyl bands was determined. Using the randomly oriented ester group as a reference, the orientation of the amide carbonyl group, and therefore the helix, has been studied[30,34,39,43]. When silicon wafers

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Table 1 Infra-red band positions of polyglutamates and polyaspartates

	Conformation	Amide I (cm^{-1})	Amide II (cm^{-1})
Polyaspartates	α_R	1659	1553
	α_L	1666	1557
	ω_L	1675	1536
	β	1636	1529
Polyglutamates	random coil	1656	1535
	α_R	1650	1546
	β (p)	1630	1530
	β (a)	1632	1530

are used as a substrate, orientation can be measured by transmission *FT*i.r. as well. In a transmission spectrum the absorption bands of the amide carbonyl group will have less intensity compared to a random helix orientation, if the α-helices are oriented perpendicularly to the surface.

PBLG in the α-helix conformation is known to have a second-order hyperpolarizability, which depends on the molecular weight of the polymer[44–46]. Terminal grafting of PBLG by initiating the polymerization from the surface with an immobilized amine results in a grafted polymer layer that is non-centrosymmetric, if the polymers exist in the α-helical conformation. Such a layer can be used to generate second harmonic radiation because of its own second-order hyperpolarizability. If the grafted polypeptides are in the α-helix conformation, it might also be possible to use the grafted layers as a polymer solvent for low-molecular-weight species, which can become oriented with respect to the oriented α-helices. Several low-molecular-weight dyes are known that do align along the helix axis of (co)polyglutamates[34].

EXPERIMENTAL

Synthesis of the grafted layers

The samples of grafted (co)polyglutamates and (co)polyaspartates were prepared by initiating the *N*-carboxy anhydrides of α-amino acids with an immobilized primary amine, as described in the previous paper[47]. Grafted copolymers were obtained by copolymerization, mixing the monomers in the desired ratio. The (co)polymers grafted onto flat surfaces were washed with dichloroacetic acid, in order to remove all free polymer. Then they were rinsed several times with chloroform to remove the dichloroacetic acid and to restore the α-helical conformation, and dried at 50°C under vacuum conditions to ensure that no solvent was left in the polymer layer.

Conformational analysis

The *FT*i.r. spectra, used for the conformation analysis, were recorded on a Bruker IFS 88 or a Mattson Galaxy 6020 spectrophotometer, with 1 cm^{-1} resolution. The polypeptides grafted onto microparticulate silica were identified using the DRIFT (diffuse reflectance infra-red Fourier transform) spectroscopic technique; the polymers grafted onto flat surfaces were studied by external reflection *FT*i.r. measurements using both parallel (p) and perpendicularly (s) polarized light; while in the case of silicon substrates transmission *FT*i.r. measurements were used as well. Changes in conformation upon heating the polymers up to 200°C were studied by comparing the *FT*i.r. spectra before and after heating.

Orientation analysis

The orientation of α-helices grafted onto flat surfaces was determined using transmission and external reflection *FT*i.r. spectroscopy with light polarized parallel to the plane of incidence, and a resolution of either 1 or 4 cm^{-1}. The external reflection *FT*i.r. spectrum of randomly oriented polymer films was calculated with a computer simulation program[48] using the transmission spectrum of a randomly oriented, free-standing film of several micrometres thickness as the input spectrum. The free-standing film was prepared by casting a polymer solution in chloroform on a glass slide or a KBr pellet, and then removing the substrate by separating the polymer film from the substrate in water, or by dissolving the KBr. The refractive index spectra of the polypeptides, necessary for the simulations, were estimated from this spectrum by the iterative procedure described by Graf *et al.*[49].

The Langmuir–Blodgett film of poly (γ-benzyl L-glutamate), consisting of 30 monolayers, was made at a temperature of 19.2°C at a pressure of 7 mN m^{-1} in a Z-type transfer. A silicon wafer, cleaned with hydrofluoric acid and then rinsed with Milli-Q water, was used as a substrate.

Interdiffusion of low-molecular-weight species

Interdiffusion experiments were carried out by submerging the grafted polymer layers for one day in a solution of *trans*-ethyl-β-apo-8′-carotenoate (Fluka) in chloroform or tetrahydrofuran. After this treatment the layer was washed four times with pure solvent to remove all excess dye molecules.

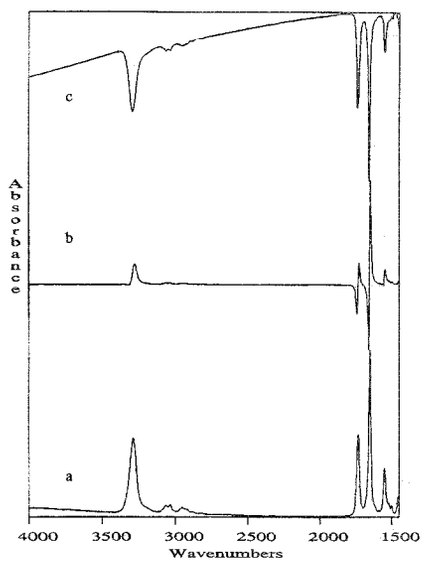

Figure 1 Simulated external reflection *FT*i.r. spectra of PBLG with different substrates: (a) free-standing PBLG film, (b) $n_s = 1.5$ (glass) and (c) $n_s = 3.8$ (silicon); film thickness 50 Å

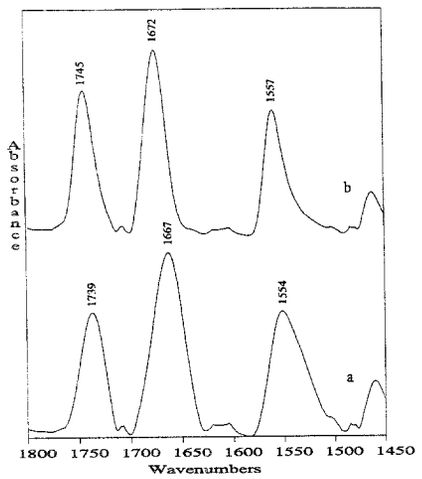

Figure 2 Example of shift in absorption band position in an *FT* i.r. reflection spectrum of P(BLA-SLA) mixed with KBr (1/1): (a) transmission spectrum and (b) reflection spectrum ($n_s = 1,5$); film thickness 50 Å

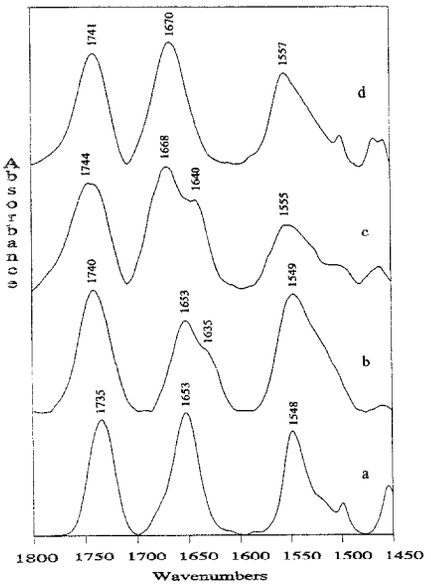

Figure 3 Polypeptides grafted onto Aerosil A200V: (a) PBLG, (b) P(MLG-SLG), (c) PBLA and (d) P(BLA-SLA). See *Table 1* for i.r. band assignments

RESULTS AND DISCUSSION

Spectrum simulations

The external reflection spectra of the polypeptides grafted onto flat surfaces cannot be compared directly to transmission spectra of randomly oriented polymers because of the optical dispersion effects inherent to the technique. Therefore the external reflection spectra were calculated from the spectrum of a free-standing film, assuming a random orientation of the polymer molecules in this film. The thickness and n_∞, the refractive index at a wavelength far from any absorption band, of the free-standing film were estimated from the interference fringes. With these values the spectrum of a free polymer film of the same thickness was calculated and compared with an experimental spectrum. The differences between the two spectra were used to modify the optical constants and a new refractive index spectrum was calculated. This was repeated in an iterative procedure as described by

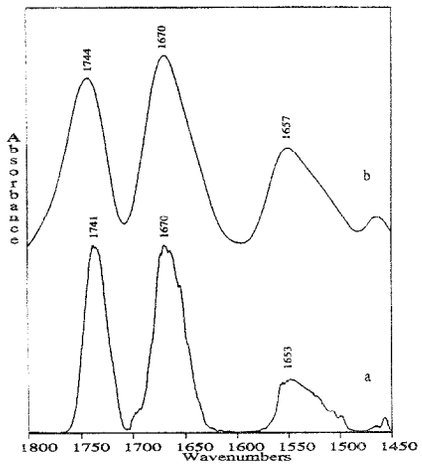

Figure 4 *FT*i.r. spectrum of grafted P(BLA-SLA) on Aerosil A200V (a) before and (b) after heating to 200°C

Graf *et al.*[49] until the calculated and experimental spectra matched completely. The resulting values were used, together with the reflection angle and refractive index of the substrate, to calculate the external reflection *FT*i.r. spectrum of a randomly oriented polypeptide film. *Figure 1* shows the calculated external reflection spectra of poly(γ-benzyl L-glutamate) on two different substrates with refractive indices of 1.5 (glass) and 3.8 (silicon)[50] respectively. Spectral simulations showed that in reflection *FT*i.r. spectra of the polymers mixed with KBr (DRIFT measurements), a shift to higher wavenumbers of several cm^{-1} occurs (*Figure 2*).

Conformational analysis

The conformations of polyglutamates and polyaspartates were determined by *FT*i.r. spectroscopy, using the results of Sasaki *et al.*[29] and Miyazawa *et al.*[39] (*Table 1*). These results had to be adjusted because of the shift to higher wavenumbers, when a reflection *FT* i.r. technique is used. The spectra of the microparticulate silica grafted (co)polyglutamates (*Figures 3a* and *3b*) showed that most polymers had taken on a right-handed α-helical conformation, which is the most stable conformation of the corresponding free polymers. This can be concluded from the positions of the amide I (1653 cm^{-1}) and amide II (1548 cm^{-1}) bands. The (co)polyaspartates grafted onto Aerosil were in the left-handed α-helical conformation (*Figures 3c* and *3d*), as was shown by the absorptions at 1668 cm^{-1} (amide I) and 1555 cm^{-1} (amide II). This left-handed α-helix is the most stable conformation for (co)polyaspartates with less than 30% long alkyl side chains[20,21,23]. The absorptions at 1635 cm^{-1} (*Figure 3b*) and 1640 cm^{-1} (*Figure 3c*) indicate the presence of some remaining β-sheet conformation as well, which is probably due to some non-grafted material at the surface. Washing experiments with dichloroacetic acid, to remove the free polymer, resulted in complete removal of all material with β-sheet conformation, and therefore it was concluded that the grafted polyglutamates were all in the right-handed α-helical conformation, while the grafted polyaspartates were in the left-handed α-helical conformation. Heating the grafted (co)polymers up to 200°C at a rate of 10°C min^{-1} in a d.s.c. experiment never showed any indication of conformational changes.

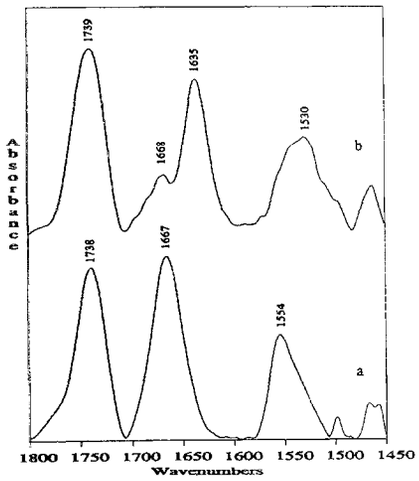

Figure 5 Change in conformation of free P(BLA-SLA) (a) before and (b) after heating to 200°C, going from a left-handed α-helix to a β-sheet

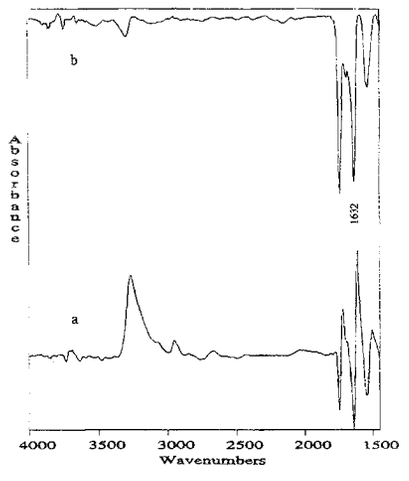

Figure 7 External reflection *FT*i.r. spectra of non-grafted polypeptides in a randomly oriented β-sheet conformation at the surface of (a) glass and (b) silicon

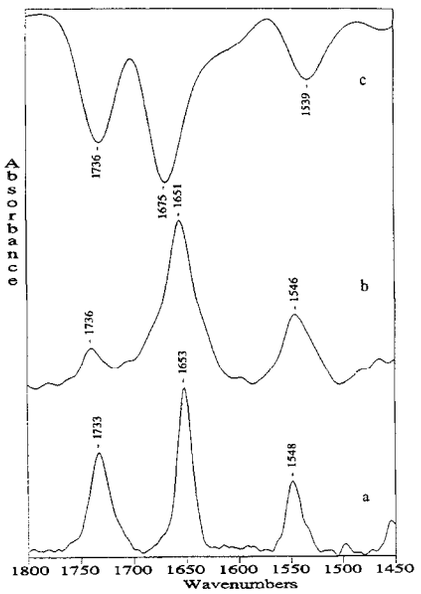

Figure 6 Polypeptides grafted onto flat surfaces: (a) PBLG (glass, external reflection), (b) P(MLG-SLG) (Si, transmission) and (c) P(BLA-SLA) (glass, external reflection). See *Table 1* for i.r. band assignments

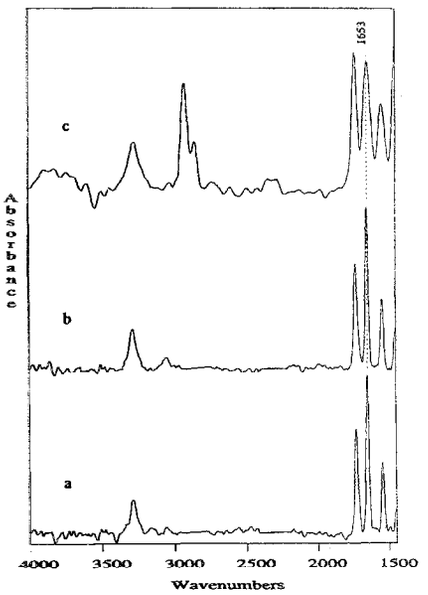

Figure 8 External reflection *FT*i.r. spectra of grafted α-helices: (a) PMLG on glass, (b) PBLG on glass and (c) P(MLG-SLG) on silicon

*FT*i.r. spectra of the polymers, before and after heat treatment, indicated no change in conformation either (*Figure 4*). None of the grafted polyaspartates showed the irreversible $\omega - \beta$ transition at around 200°C, reported by Tsujita *et al.*[27] for free polyaspartates, and shown in the *FT* i.r. spectrum in *Figure 5* for free poly(β-benzyl L-aspartate-*co*-β-stearyl L-aspartate). The absence of the change into a β-sheet conformation of the grafted (co)polyaspartates could be explained by the fact that, as the chains are fixed to the surface by the same end, the formation of the most stable antiparallel β-sheet structure is prevented.

The conformations of (co)polyglutamates and (co)polyaspartates grafted onto glass slides or silicon wafers were determined by *FT*i.r. measurements as well (*Figure 6*). For glass slides the external reflection technique was used, with an angle of incidence of 80°, while for the silicon wafers, which are transparent for infra-red light, transmission experiments could be used too. As with the polypeptides grafted onto microparticulate silica, no differences in the conformations were observed for the polyglutamates grafted onto flat surfaces compared to those of the free chains. They were all in a right-handed α-helical conformation, with the amide I band at 1653 cm^{-1} and the amide II band at 1548 cm^{-1}. The absorption bands at 1675 and 1539 cm^{-1} indicate that the grafted poly(β-benzyl L-aspartate-*co*-β-stearyl L-aspartate) is in a left-handed ω-helical conformation. This means that this polymer has probably more than 30% long alkyl side chains[20,21,23].

Orientation analysis

Although the measured *FT*i.r. spectra all allowed a good conformational analysis, the determination of the orientation of the grafted chains proved to be much more difficult. Orientation of the helices can be derived from the orientation of the amide I band, caused by the

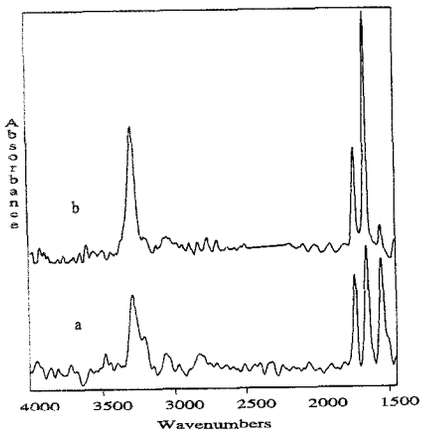

Figure 9 *FT*i.r. spectra of a Langmuir–Blodgett film of PBLG: (a) external reflectance (p) and (b) transmission

carbonyl bond of the amide, lying in the direction of the helix axis. The absorbance of this group will increase when the helices are more parallel to the polarization direction of the infra-red light. By comparing the ratio of the absorbances of the amide I band and of the ester band to the ratio of the same bands in a spectrum of a polymer film that is known to be randomly oriented, the orientation of the grafted polymers at the surface can be determined. The ester group is randomly oriented in all cases, and its absorbance can therefore be used as a reference[43].

As expected, the *FT*i.r. spectra of the (co)polyglutamates and (co)polyaspartates grafted onto microparticulate silica did not show any marked differences compared to the transmission spectra of free randomly oriented polymers in KBr.

The external reflection spectra of the polymers grafted onto flat surfaces, directly after polymerization, were identical to the calculated spectra. The polypeptides were usually in a β-sheet conformation, which can be seen from the amide I absorption at 1632 cm^{-1} (*Figure 7*). These β-sheets could be removed by washing with dichloroacetic acid, indicating that they are not covalently attached to the surface. The relatively thick β-sheet layers are therefore not supposed to show an overall orientation, as confirmed by the *FT*i.r. spectra. After washing with dichloroacetic acid, only the grafted polypeptides remained at the surface. The external reflection spectra of these grafted polymers were different from the calculated spectra. *Figure 8* shows the external reflection spectra of three polyglutamates grafted onto the surface of glass (*Figures 8a* and *8b*) and silicon (*Figure 8c*). The absence of any negative or 'derivative' adsorption bands as seen in the calculated spectra (*Figure 1*) could be an indication of orientation of the helices. This was confirmed by comparing the external reflection spectrum of a Langmuir–Blodgett multilayer of poly(γ-benzyl L-glutamate) on a silicon wafer. In a Langmuir–Blodgett layer the α-helices are known to be oriented parallel to the surface[51,52], and this orientation caused big differences in the *FT*i.r. spectra (*Figure 9*). Both the change in intensity ratio of the amide I and carbonyl ester band and the change in the external reflection spectra indicate that the grafted helices are bent towards the surface in such a way that the overall orientation might be more or less comparable to a Langmuir–Blodgett layer.

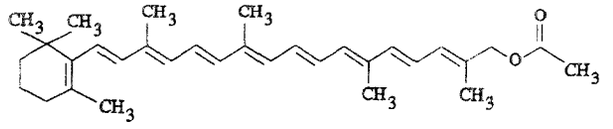

Figure 10 *Trans*-ethyl-β-apo-8′-carotenoate

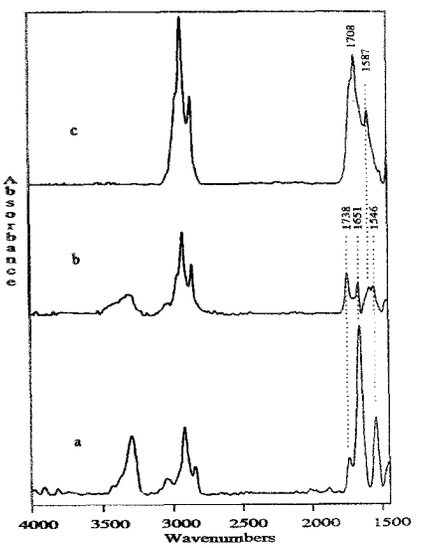

Figure 11 Transmission *FT*i.r. spectra of P(MLG-SLG) grafted onto a silicon wafer (a) before and (b) after interdiffusion of *trans*-ethyl-β-apo-8′-carotenoate. (c) *FT*i.r. spectrum of *trans*-ethyl-β-apo-8′-carotenoate

Interdiffusion of low-molecular-weight species

Interdiffusion experiments caused a big change in the *FT*i.r. spectra of the grafted layers. This is shown by the interdiffusion of a low-molecular-weight dye, *trans*-ethyl-β-apo-8′-carotenoate (*Figure 10*), into grafted poly(γ-methyl L-glutamate-*co*-γ-stearyl L-glutamate) on a silicon wafer. From infra-red measurements on Langmuir–Blodgett films of this system, *trans*-ethyl-β-apo-8′-carotenoate is known to orient parallel to the glutamate helix[37]. *Figure 11a* shows the transmission *FT*i.r. spectrum of the grafted polymer after washing with dichloroacetic acid and chloroform. The amide I band is much larger than expected for a randomly oriented polyglutamate film, which indicates that helices are lying more or less flat on the surface. *Figure 11c* shows the transmission spectrum of *trans*-ethyl-β-apo-8′-carotenoate. *Figure 11b* shows the transmission *FT*i.r. spectrum of the same sample after interdiffusion with *trans*-ethyl-β-apo-8′-carotenoate. The position of the amide I band remains at 1651 cm^{-1}, indicating that the polymer is still in the α-helical conformation. The absorbance of the amide I band has become less than that of the carbonyl band of the ester, which remains the same, meaning that the helices are now oriented more perpendicularly to the surface. The presence of the dye in the grafted layer is shown in *Figure 11b* by the larger alkyl absorbance and the absorption band at 1587 cm^{-1}, originating from the dye molecule.

CONCLUSIONS

There is no effect of the grafting of (co)polyglutamates and (co)polyaspartates on their most stable secondary

structure. When all free polymers are washed away, the grafted polymers take on the same conformations as the corresponding free polymers, which is easily detected with *FT*i.r. measurements. The only effect of grafting is the prevention of the formation of a β-sheet structure upon heating, probably caused by the forced parallel alignment of the helices.

Orientation measurements were more difficult, but there is an indication that the grafted (co)polyglutamates and (co)polyaspartates in an α-helical conformation are bent towards the surface. Interdiffusion of a low-molecular-weight polar species can cause the helices to erect.

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