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Chiral memory and self-replication study of porphyrin and bilirubin aggregates formed on polypeptide matrices

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Porphyrin heteroaggregates composed of *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphinatocopper(II) and *meso*-tetrakis(4-sulfonatophenyl)porphyrin formed in the presence of a polyglutamic matrix possess chiral memory and an ability to self-replicate their supramolecular structures. By means of electronic circular dichroism spectroscopy, it has been shown that the self-replication process is not influenced by changes in pH or ionic strength. The average molecular weight of the polyglutamic matrix used for porphyrin aggregate preparation plays a crucial role with regard to the form of the resulting CD spectrum. In the second part of our study, a complex composed of polylysine and bilirubin as a model system for a structured homoaggregate formed on the chiral matrix has been tested for chiral memory phenomena. The results indicate that the bilirubin homoaggregate shows chiral memory.

Keywords: porphyrins; chiral memory; self-assembly; self-replication; bilirubin; ECD

Introduction

Binary complexes of polypeptide matrices and porphyrins have been the subject of many detailed studies (*1–7*). They can be used as model systems for the induction of chiral information from the chiral matrix to achiral guest molecules. The porphyrin core has a plane of symmetry and is therefore achiral; however, it can gain chirality through the formation of higher supramolecular structures. Two main types of polypeptide–porphyrin systems have been studied: positively charged polypeptide matrices that interact with anionic porphyrins and negatively charged polypeptide matrices that interact with cationic porphyrins. Anionic *meso*-tetrakis (4-sulfonatophenyl)porphyrin (TPPS) and *meso*-tetrakis (4-benzoato)porphyrin (TPPC) serve as porphyrin partners in the formation of binary complexes with positively charged polylysine (*1*, *6*, *8*). Cationic *meso*-tetrakis(α-trimethylammonio-*p*-tolyl)porphyrin (TATP), *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (T4), and *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphinatocopper(II) (CuT4) serve as partners in the formation of binary complexes with negatively charged polyglutamate (*1–5*, *7*). Organised chiral architectures of porphyrin units bound to polypeptides exhibit induced circular dichroism bands in the Soret region. The conformations of the binary complexes are dependent on the polypeptide matrix and their structures can therefore be easily varied by changing the conformation of the matrix.

The complex of polyglutamic acid and CuT4 has been well described (*2*, *4*, *5*, *7*) through the application of electronic circular dichroism (ECD) spectroscopy. At low pH, when the polyglutamic chain adopts an α-helical conformation, induced circular dichroism (ICD) in the form of a characteristic doublet in the Soret region is observed, indicating that CuT4 interacts with the polyglutamic matrix and creates a chiral architecture. In alkaline media, in which polyglutamic acid is almost fully deprotonated and adopts a structure similar to that of polyproline II (PPII), also referred to as the random coil conformation, no evidence of induction of chiral information to the porphyrin molecules is observed. In this case, the porphyrin units are bound to polyglutamate; however, their structure is irregular and does not give rise to ICD bands in the Soret region.

Unlike relatively simple binary complexes, ternary aggregates composed of two oppositely charged guest molecules and a polymeric matrix represent more complex systems. Purrello et al. have published a number of papers dealing with ternary complexes consisting of a chiral template and two oppositely charged porphyrins (*2*, *5*, *7*, *9–12*). It was proved that metallation of the cationic or anionic porphyrin significantly influences the dimensions and spectral properties of the resulting aggregate (*12*). The most promising results were obtained using a polyglutamic matrix, cationic porphyrin CuT4, and anionic porphyrin

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TPPS (*5*). Such a supramolecular complex can be prepared through a self-assembling process by simple addition of the anionic porphyrin to the preformed binary complex at pH ≈ 3.6 (*7*), at which the polyglutamic matrix adopts an α-helical conformation. The structure of this complex originates from the geometry of polyglutamic acid in α-helical conformation, as is evident from the fact that the mirror image CD spectrum is obtained when poly-D-glutamate is used instead of poly-L-glutamate (*9*). Porphyrin heteroaggregation has also been studied using phenylalanine as the chiral template (*2*, *10*, *11*). This amino acid allows the formation of a chiral porphyrin heteroaggregate when it is used at a concentration in excess of 1×10^{-3} M. The biggest advantage of a phenylalanine template in comparison with a polyglutamic matrix is the possibility of removing the matrix by ultrafiltration.

Once formed, the porphyrin heteroaggregates are remarkably inert and become independent of the matrix. The porphyrin assemblies have the capacity for chiral memory, that is, they retain their structure after a change in the conformation of the matrix on increasing the pH to around 12 or upon the addition of the opposite matrix (*7*, *9*).

The most interesting phenomenon observed for porphyrin heteroaggregates is their self-replication ability (*2*, *10*, *11*). This phenomenon is manifested as an ability of supramolecular systems to form structures with an identical arrangement when monomeric non-ordered units are added. Repeated additions of equimolar aliquots of cationic and anionic porphyrins to a solution in which a chiral porphyrin heteroaggregate is present result in an intensity increase of the ICD bands characteristic of the chiral porphyrin structure that is proportional to the added amount of the porphyrins. The process is almost 100% enantiospecific and occurs even when the concentration of the chiral template is subpicomolar (*10*). In experiments using a chiral porphyrin aggregate, removal of the phenylalanine matrix clearly showed that the enantiospecific autoreplication ability of the porphyrin assembly is independent of the matrix.

The properties of the porphyrin heteroaggregates described above indicate that these substances can be used as model systems to study the storage and transfer of chiral information in Nature.

In contrast to polypeptide–porphyrin systems, which have been the subject of many detailed studies, a system consisting of bilirubin and a polylysine matrix has not hitherto been tested with regard to chiral memory phenomena. Bilirubin is a linear tetrapyrrole dicarboxylic acid found characteristically in mammals, where it is produced by catabolism of heme. It consists of two conjugated dipyrrole halves and the variety of possible conformations depends on the spatial arrangement of the halves. In solution, bilirubin exists as an equimolar mixture of two isoenergetic conformers with M and P helicities, called the 'ridge-tile' conformation, which are stabilised by six intramolecular hydrogen bonds (*13*). Self-aggregation of bilirubin anions, which leads to weak intermolecular electric dipole coupling, has been proposed previously (*14*, *15*, *16*). The bilirubin monomer or aggregates with a defined orientation of the transition dipole moments are preferentially recognised by a variety of optically active molecules, such as polypeptides (*15*) or proteins (*17*), as has been investigated by CD spectroscopy.

From the literature, it is known that right- and left-handed α-helices of poly-L-lysine and poly-D-lysine preferentially select bilirubin self-aggregates with transitition dipole moments with opposite screw senses (*15*). Enantioselective complexation does not occur when the polypeptide is in the PPII conformation.

The goals of our study were to determine the impact of the length of the polyglutamic matrix on the chirogenesis of cationic and anionic porphyrin complexes, to test the self-replication ability of a chiral porphyrin heteroaggregate under conditions different from those that are optimal for ternary complex preparation, and to test bilirubin homoaggregates formed in the presence of polylysine for chiral memory.

Experimental

Poly-L-glutamic acid with average molecular weights of 1500–3000, 13,000, 17,000, 70,500 and 97,800 g mol^{-1} and poly-D-glutamic acid with average molecular weights of 14,500 and 38,000 g mol^{-1} in the form of the sodium salt, as well as poly-L-lysine with an average molecular weight of 55,200 g mol^{-1} and poly-D-lysine with an average molecular weight of 44,100 g mol^{-1} were purchased from Sigma and used without further purification. *meso*-Tetrakis(4-sulfonatophenyl)porphyrin (TPPS) was obtained in purity exceeding 98% from the Department of Peptide Chemistry, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague and used without further purification. *meso*-Tetrakis(*N*-methylpyridinium-4-yl)porphinatocopper(II) (CuT4) was prepared by a routine metallation procedure from *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphyrin purchased from Porphyrin Systems. The structures of both porphyrins are shown in Figure 1. Bilirubin IX of purity about 99% was purchased from Frontier Scientific and was used without further purification. The structures of the two isoenergetic conformers of bilirubin are shown in Figure 2.

The binary complex of a polyglutamic matrix and CuT4 was prepared at pH ≈ 4.5. After formation of the binary complex, the pH was adjusted to 3.5 and TPPS was added. All pH adjustments were made with citrate buffer. In all of the prepared ternary complexes, the concentration of polyglutamic acid, expressed per amino acid residue, was 2×10^{-4} M, and the concentrations

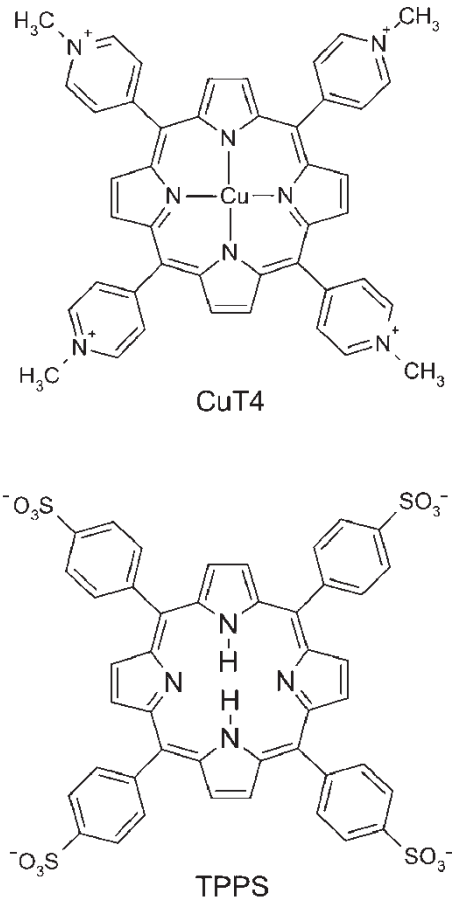

Figure 1. Structures of *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphinatocopper(II) (CuT4) and *meso*-tetrakis(4-sulfonatophenyl)porphyrin (TPPS).

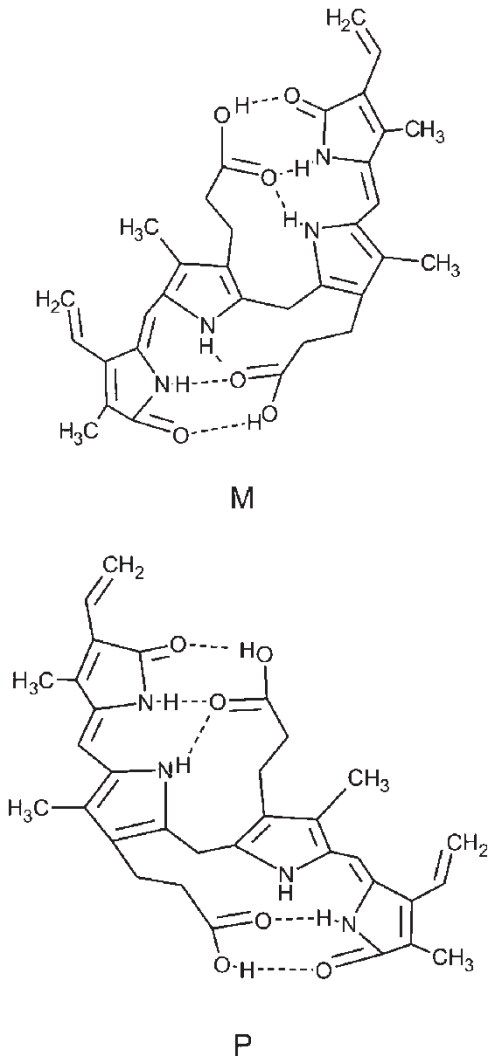

Figure 2. Structures of bilirubin 'ridge-tile' conformers with M and P helicities.

of the cationic and anionic porphyrins were 4×10^{-6} M. Ternary complex solutions with different average molecular weights of the polyglutamic matrix were prepared using the same primary solutions to minimise dilution errors. Complexes of polypeptide and bilirubin were prepared at pH 10.8 to achieve bilirubin solubility. In all bilirubin–polylysine systems, the concentration of polylysine, expressed per amino acid residue, was 8×10^{-4} M, and the concentration of bilirubin was 8×10^{-5} M. All measurements of solutions containing bilirubin were performed 30 min after their preparation. Doubly-distilled water was used throughout. Electronic circular dichroism (ECD) measurements were carried out on a Jasco J-810 spectropolarimeter.

Discussion

Effect of the matrix chain length

Figure 3 shows CD spectra of the ternary complexes obtained when poly-L-glutamic acid matrices with average molecular weights of 1500–3000, 13,000, 17,000, 70,500 and 97,800 g mol^{-1} were used. Remarkable differences can be seen in both the shape and intensity of the spectra. The spectra of the complexes with matrices of molecular weight up to 17,000 g mol^{-1} feature spectral bands situated in approximately the same positions, differing only in the ICD band intensity (curves a, c, and d). For the polyglutamic matrices with distinctly longer chains of 70,500 and 97,800 g mol^{-1} (curves f and g), the spectral shape is completely different with a dominant couplet having a negative maximum at 424 nm and a positive maximum at 440 nm. The same trend is observed when poly-D-glutamic acid is used instead of poly-L-glutamic acid. The use of a poly-D-glutamic matrix with an average molecular weight of 14,500 g mol^{-1} (curve b) results in a spectral shape almost perfectly symmetrical to that obtained when poly-L-glutamic acid with an average molecular weight of 13,000 g mol^{-1} is used. The ternary complex obtained using poly-D-glutamic acid with an average molecular weight of 38,000 g mol^{-1} (curve e)

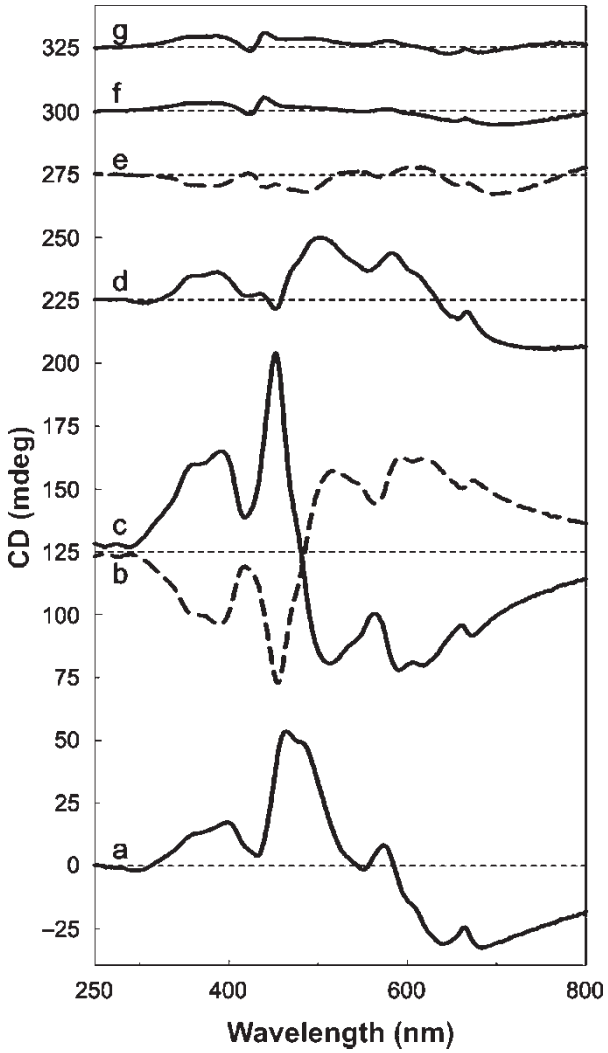

Figure 3. CD spectra of CuT4–TPPS aggregate (4×10^{-6} M each) formed in the presence of poly-L-glutamate (full lines, 2×10^{-4} M) and poly-D-glutamate (dashed lines, 2×10^{-4} M) with average molecular weights of (a)1500–3000, (b)14,500, (c)13,000, (d)17,000, (e)38,000, (f)70,500 and (g)97,800 g mol^{-1}. Citrate buffer (5×10^{-3} M), pH ≈ 3.6. CD spectra (b)–(g) are off-set with zero lines dotted.

exhibits ICD bands characteristic of a ternary complex, but their intensities are significantly lower, in accordance with the observation when poly-L-glutamic acid was used.

It is obvious that polyglutamic matrix length is a crucial factor for matrix-assisted self-assembly of cationic and anionic porphyrins. According to our results presented in Figure 3, the most intense ICD bands are observed when a polyglutamic matrix with an approximate molecular weight of 13,000 g mol^{-1} (~100 glutamic units) is used. For longer polyglutamic matrices, the intensity of the characteristic ICD bands decreases. For matrices containing more than about 500 glutamic units (average molecular weights 70,500 and 97,800 g mol^{-1}), the spectrum features only a couplet typical of a binary complex formed between the polyglutamic matrix and CuT4. The effect of the matrix average molecular weight can also be observed by UV–Vis absorption. It has been demonstrated (*1*) that the porphyrin supramolecular structure chirogenesis is manifested in a broader absorption and a decreased intensity of the porphyrin signals in the Soret region (hypochromic effect). Figure 4 shows the absorbance of the ternary system at 434 nm in comparison with the absorbance of the binary complex at 428 nm when polyglutamic matrices with different average molecular weights are used. For the 'optimal' polypeptide chain length (average molecular weight ≈13,000 g mol^{-1}), the absorbance value at 434 nm typical of the free protonated form of TPPS is about three times lower than when the polyglutamic matrix with an average molecular weight of 97,800 g mol^{-1} is used. This suggests that a significantly lower proportion of the anionic porphyrin molecules interacts with the cationic porphyrin units when a long matrix chain is used. In this case, the main components in the solution are the protonated form of TPPS, which does not interact with the matrix, and CuT4 bound to the polyglutamic matrix, and the chirogenesis of the porphyrin supramolecular structure does not proceed. Both ECD and UV–Vis absorption data indicate that a polyglutamic matrix with approximately 100 glutamic units seems to be optimal for the preparation of the porphyrin chiral supramolecular structure.

For binary complexes, the dependence of the absorbance in the Soret region on the average molecular

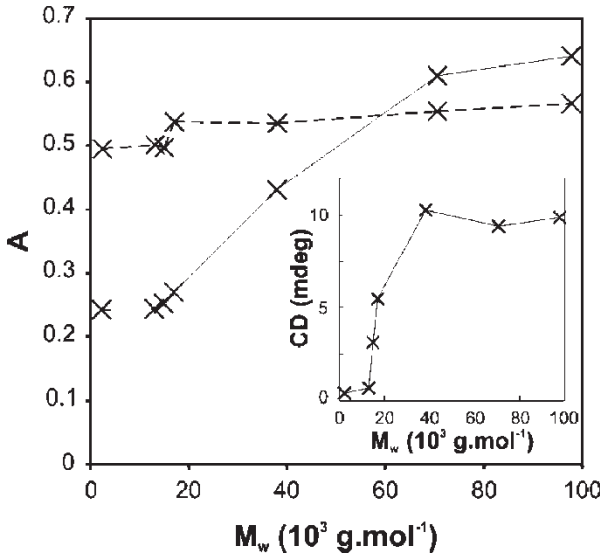

Figure 4. Dependence of the absorbance in the Soret band of CuT4–TPPS aggregate (full line, 4×10^{-6} M each, 434 nm) and CuT4 (dashed line, 4×10^{-6} M, 428 nm) on the average molecular weight of the polyglutamic matrix used (2×10^{-4} M). Inset: Dependence of CD of CuT4 (4×10^{-6} M, 439 nm) on the average molecular weight of the polyglutamic matrix present (2×10^{-4} M). Citrate buffer (5×10^{-3} M), pH ≈ 3.6.

weight of the matrix is different (Figure 4). When using polypeptide chains of different molecular weights, the absorbances show very small differences in intensity, while the intensities of the CD bands differ significantly and generally increase with increasing molecular weight of the matrix. This behaviour is to be expected because the porphyrin molecules are bound to the polypeptide chain by the same mechanism for the matrices of all lengths. The various intensities of the ICD bands are believed to be caused by slight distinctions in the geometries of the polyglutamic chains with different molecular weights, which are transferred to the spatial organisation of the CuT4 cationic porphyrin units.

Self-replication study

The self-replication process was studied at pH ≈ 3.6 and under conditions of low ionic strength, which are optimal for ternary complex chirogenesis (*7*). Our task was to test the self-replication ability when the pH or ionic strength was out of the value range that implicates ternary complex formation.

The first step of this study was to test the self-replication ability of the ternary complex of CuT4, TPPS, and PLGA with an average molecular weight of 13,000 g mol^{-1} at pH ≈ 12 by adding equimolar amounts of the cationic and anionic porphyrins (Figure 5). At this pH, the polyglutamic matrix adopts the PPII structure. After the pH was adjusted to the desired value by adding KOH to the solution of the preformed ternary complex, the concentrations of both porphyrins CuT4 and TPPS were increased by 2 × 10^{-6} M at each step (half of the original concentration in the ternary complex solution). A proportional increase in the intensities of the ICD bands was observed after each step, indicating the occurrence of a self-replication process. We conclude that the self-replication ability of the porphyrin supramolecular complex is not affected by conditions of high pH.

The next experiment was carried out to test the self-replication ability in alkaline medium when the polypeptide matrix was removed. The ternary complex was prepared using PLGA matrix with an average molecular weight of 13,000 g mol^{-1} and the pH of the solution was set at around 12 using KOH solution. At each step, half of the sample volume was removed and replaced by a solution containing all components other than the matrix. The aim was to halve the matrix concentration at each step and to keep the concentrations of all other components constant. This matrix-removing procedure was repeated six times rendering the polyglutamate concentration in the final sixth step 1/64 of the initial concentration at the beginning of the experiment. Decreased intensity of the ICD bands (Figure 6) indicates that only a limited proportion of the newly added porphyrin molecules participate in the self-replication process. Nonetheless, it is evident that to some

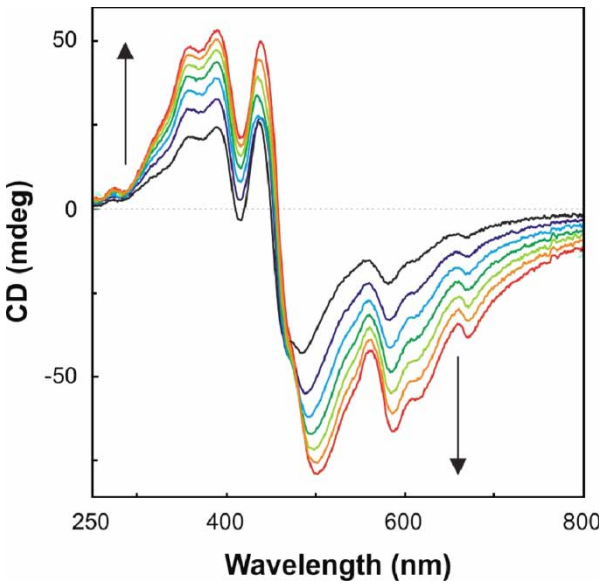

Figure 5. CD spectra of CuT4–TPPS aggregate (4 × 10^{-6} M each) formed in the presence of poly-L-glutamate (2 × 10^{-4} M) with an average molecular weight of 13,000 g mol^{-1} after six additions of CuT4 and TPPS (at each step, the concentration increased by 2 × 10^{-6} M), pH ≈ 12.

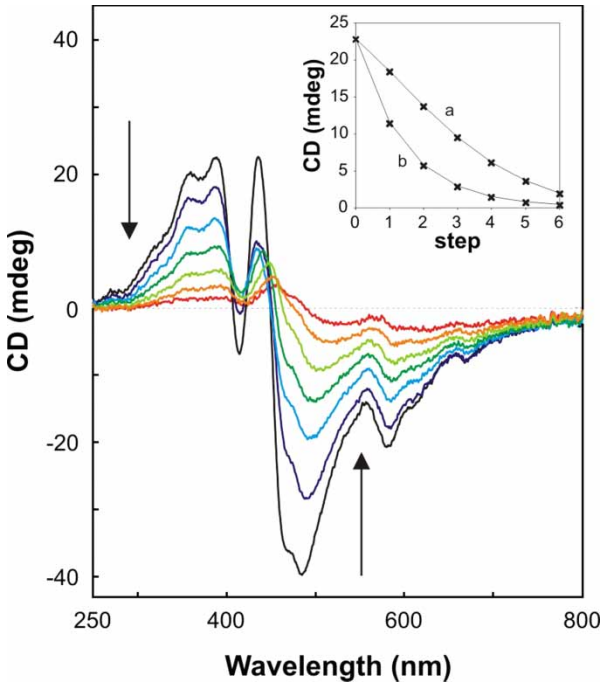

Figure 6. CD spectra of CuT4–TPPS aggregate (4 × 10^{-6} M each) formed in the presence of poly-L-glutamate (2 × 10^{-4} M) with an average molecular weight of 13,000 g mol^{-1} after six steps in which half of the sample was replaced by a solution containing all components other than the matrix, pH ≈ 12. Inset: CD intensity (390 nm) of (a) the measured solution and (b) a system with no self-replication ability.

extent the supramolecular replication is operating. When we compare the intensities of the ICD bands of the measured sample during each of the steps with the theoretical values when the ternary complex concentration is determined only by the initial concentration and is halved at each step, we can see that the measured intensities are significantly higher.

In the next step, we tested the influence of increased ionic strength on the self-replication ability of the porphyrin supramolecular complex. As in the previous experiments, a ternary complex based on PLGA with an average molecular weight of 13,000 g mol^{-1} was used. Ionic strength was set using KCl solution. The concentration of KCl in the ternary complex solution was 5×10^{-2} M and the pH was around 12. Figure 7A shows CD spectra of the ternary complex obtained when equimolar amounts of CuT4 and TPPS were added in three steps. At each step, the concentrations of both porphyrins CuT4 and TPPS were increased by 2×10^{-6} M. Increased intensity of the ICD bands characteristic of the porphyrin supramolecular complex indicates the occurrence of self-replication. This behaviour is in contrast to the effect of high salt concentration when the salt is added to the binary complex solution before the anionic porphyrin is added, which results in zero CD signal (Figure 7B). This experiment proves that high salt concentrations do not influence the self-replication ability of the porphyrin chiral aggregates.

According to the performed experiments, the self-replication ability of porphyrin aggregates is maintained over a wide range of physicochemical conditions in solution. In contrast to ternary complex preparation, which is extremely pH and ionic strength-sensitive, the porphyrin complex formed with template-imprinted structure is extremely stable and its capacity for self-replication is only slightly affected by changes in pH and ionic strength. The robustness of the self-replication process makes this technique very promising as a potential means of chiral amplification that would be capable of detecting very low concentrations of chiral analytes.

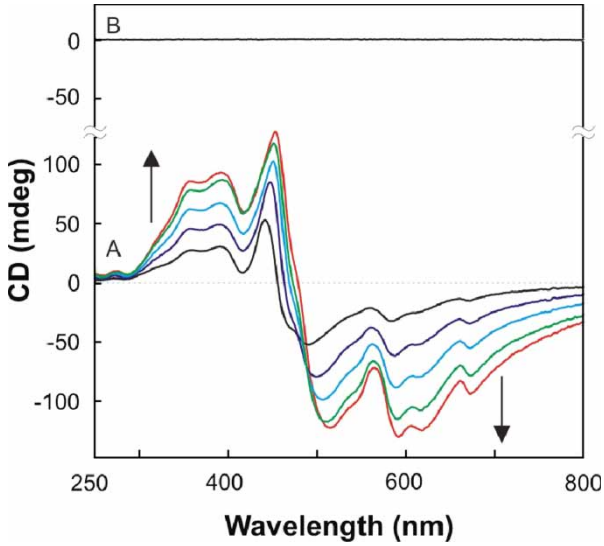

Figure 7. CD spectra of CuT4–TPPS aggregate (4×10^{-6} M each) formed in the presence of poly-L-glutamate (2×10^{-4} M) with an average molecular weight of 13,000 g mol^{-1} after several additions of CuT4 and TPPS (at each step, the concentration increased by 2×10^{-6} M) in the presence of KCl (5×10^{-2} M) added (A) after and (B) before the initial ternary complex formation.

Polylysine–bilirubin system

Figure 8 shows CD spectra of the binary complex of bilirubin and polylysine obtained when poly-D-lysine or poly-L-lysine was used as the polypeptide matrix. For both matrices, the CD spectrum exhibits intense bands in the region 220–300 nm attributable to the polylysine matrix and ICD bands attributable to bilirubin with a maximum of the major band at 452 nm. It is therefore possible to follow the signals of the polypeptide matrix and bilirubin in distinct spectral regions. The spectra clearly demonstrate that the structure of the polylysine–bilirubin complex originates from the geometry of the matrix, as is readily apparent from the symmetry of the CD bands of the complex when opposite matrices are used.

The capacity for chiral memory of the polylysine–bilirubin system was tested by adding the opposite matrix to the preformed binary complex solution. The addition was carried out in two steps: in the first step, the concentration ratio of the original and the newly added matrix was 1:1, and then in the second step the addition of the new matrix was repeated, making the concentration ratio 1:2. Figure 9A shows the CD spectra of the binary complex of poly-L-lysine and bilirubin before and

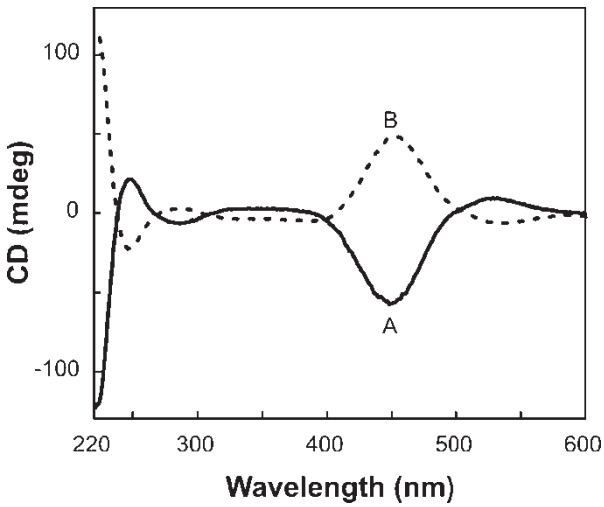

Figure 8. CD spectra of bilirubin (8×10^{-5} M) in the presence of (A) poly-L-lysine (8×10^{-4} M) and (B) poly-D-lysine, pH ≈ 11.

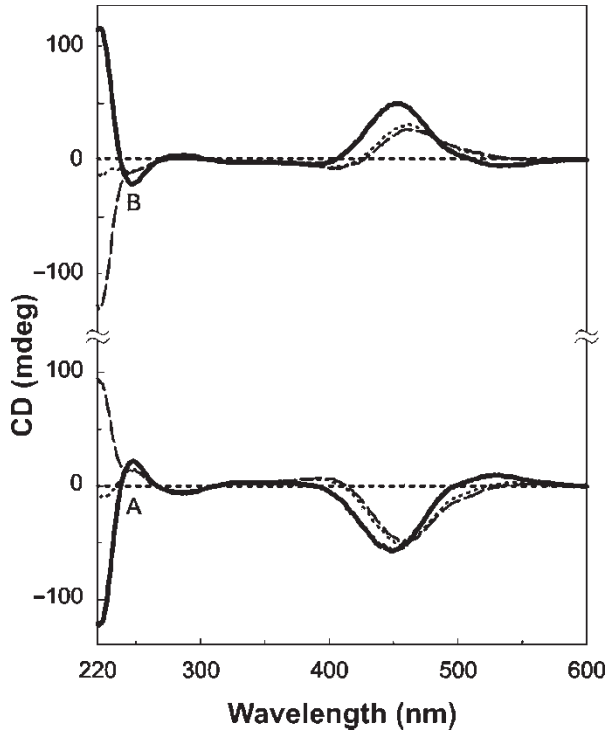

Figure 9. CD spectra of bilirubin (8×10^{-5} M) in the presence of (A) poly-L-lysine (8×10^{-4} M) and (B) poly-D-lysine before (full line) and after opposite matrix additions, matrix ratio 1:1 (dotted line) and matrix ratio 1:2 (dashed line), pH ≈ 11.

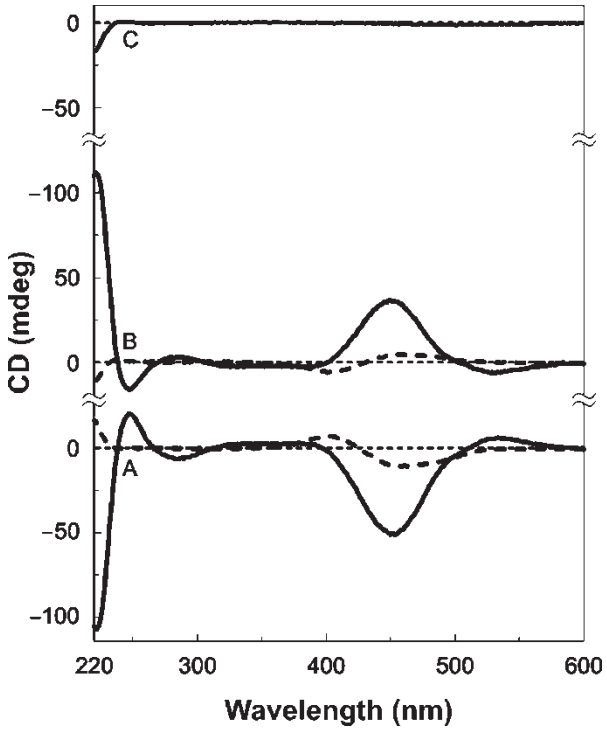

Figure 10. CD spectra of bilirubin (8×10^{-5} M) in the presence of (A) poly-L-lysine (8×10^{-4} M) and (B) poly-D-lysine before (full line) and after (dashed line) a pH jump from pH ≈ 11 to 5 and (C) of a sample containing the same components after addition of bilirubin to the matrix after the pH jump to pH ≈ 5.

after the addition of poly-D-lysine. After poly-D-lysine is added, the intensity of the major ICD band decreases to approximately two-thirds of the original value and the position of the maximum is shifted from 453 to 461 nm. The CD signal in the region 220–300 nm is close to zero because of the 1:1 ratio of poly-L-lysine and poly-D-lysine in the solution. Further addition of poly-D-lysine inverts the signal in the region 220–300 nm to a negative value but the previous signal of bilirubin is unchanged. The changes in the major band are accompanied by a slight change in the side bands on either side of the major band. These changes are believed to be caused by a small change in the angle between the dipyrrole groups of the bilirubin molecules in the conjugate. We conclude that the structure of the bilirubin units in the complex changes slightly after the opposite matrix is added, but that the main geometry character derived from the polypeptide matrix is retained. The excess of the opposite matrix after the second addition is confirmed by inversion of the ICD band at 222 nm. Figure 9B shows the CD spectra of poly-D-lysine and bilirubin before and after the addition of the opposite matrix. Inverse spectral shape with respect to the previous spectra and the same spectral changes after addition of the opposite polylysine matrix prove chiral memory of the polylysine–bilirubin conjugate.

Changing the matrix conformation is another method for assessing the chiral memory of the bilirubin conjugate. Figure 10A shows the CD spectra of the complex of poly-L-lysine and bilirubin before and after a pH jump from pH ≈ 11 to 5, which is accompanied by a change in the secondary structure of the polypeptide matrix from the α-helical conformation to the PPII structure, as is apparent in the region 220–300 nm. This conformational change is accompanied by a decrease in intensity of the major ICD band to approximately one-fifth of the original intensity and an increase in the intensity of the side band at 405 nm. The observed slight change of bilirubin signal caused by a decrease in pH was observed previously (*18*) and was explained by increasing of the angle between dipyrrole chromophores. The same behaviour is observed when using poly-D-lysine as the matrix (Figure 10B). The structure of the bilirubin complex is considered to be disrupted to some extent after the conformational change, but nonetheless some chiral information is retained. It should be noted that when bilirubin is added to the polylysine matrix in PPII conformation, which is realised by adjusting the pH of the solution to around 5, the spectrum exhibits no ICD bands (Figure 10C).

According to our experiments, the binary system of polylysine and bilirubin exhibits chiral memory effects. In view of the great importance of bilirubin in many biochemical processes, further investigations aimed at studying the interactions of bilirubin with bioactive polymers would seem appropriate.

Acknowledgements

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