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Estradiol binding synthetic polypeptides

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We have synthesised polypeptides that mimic the binding properties of natural receptors with high affinity and selectivity towards the steroid hormone estradiol, performing a template polymerisation in aqueous medium and without creating rigid structures.

Selective recognition systems are usually created in synthetic polymers obtaining a three-dimensional structure that has been shaped to match the template, after its removal. As a consequence, artificial systems of recognition are available for nucleotides,[1] inorganic ions,[2] amino acids[3,4] and herbicides.[5] We employed the template polymerisation method to synthesise oligopeptides that were templated with the steroid hormone estradiol. The application of this technique is completely new because until now the literature only gave examples on cross-linked organic macroporous polymers, whereas we performed the polymerisation in aqueous medium without the formation of rigid structures. The initial idea was that an amino acid mixture could generate polypeptidic systems, thus establishing non-covalent bonds with molecular species present in the polymerisation mixture. Polypeptides are structures that are well suited for the design of systems with molecular recognition properties, above all for the large structural heterogeneity in the amino acid lateral chain that gives rise to a great variety of chemical properties and non-covalent interactions useful in template polymerisation.

The amino acid composition of the mixtures that we polymerised is similar to bovine serum albumin. Two different amino acid mixtures were polymerised: the first—branched mixtures— contained branched monomers (Gly, Ala, Leu, Pro, Phe, Tyr, Trp, Ser, Met, Arg, His, Lys, Asp, Asn, Gln, Glu, Cys-Cys), the second—linear mixtures— contained the same amino acids apart from Lys, Asp, Glu, Cys-Cys and were richer in Leu and Gly. This enabled us to study the binding properties of both cross-linked and linear structures. Furthermore, for each mixture the polymerisation was performed in the presence (template mixtures) and in the absence (blank mixtures) of estradiol to compare the binding properties, and were performed for different reaction times (1, 3 and 30 h) to evaluate the influence of this variable on the binding constants.[6]

To remove the estradiol we used anion exchange chromatography on a DEAE-Sephacel column.[7]

The concentration of the oligopeptides was determined by spectrophotometric measurements, and the recoveries were between 94 and 97%.

After purification of the mixtures, we characterised the oligopeptides by determining the average molecular weights by HPLC using an Alltech Macrosphere GPC 60 A 7 μm (250 mm × 4.6 mm).[8] The average M_w was between 2000 and 6000 Da, increasing with the polymerisation time, and so, considering an average amino acid M_w of 130 Da, the degree of polymerisation was between 15 and 46, with an average value of 30.

The spectrophotometric titration of the template oligopeptides with estradiol performed at 274 nm shows that the stoichiometry of binding is close to 1 : 1, but an evaluation of the binding constant was not possible due to the absence of curvature in the equivalence region.

To evaluate the binding affinity of the oligopeptides we used immunocompetition where the oligopeptides competed with an immobilised anti-estradiol antibody for tritium labelled estradiol. The anti-estradiol antibody was immobilised into the wells of microtitre plates previously coated with goat anti-rabbit IgG antibodies, according to the literature.[9] The measurement of estradiol bound to the antibody in the presence of the oligopeptides allowed us to determine the binding constants K (by fitting a proper mathematical equation to the experimental data) of the oligopeptide mixtures. This defines the ratio between the binding of the tritium labelled estradiol with the antibody in the presence of the oligopeptides and without the oligopeptides, as a function of the binding constant of the antibodies for estradiol, the concentration of the solid phase, binding site antibodies, the tritium labelled estradiol concentration, the concentration of the oligopeptides and the binding constant of the oligopeptides for the estradiol. The first two parameters were determined in separate experiments as reported in the literature,[9] thus, knowing the tritium labelled estradiol concentration and the concentration of the oligopeptides we could obtain the binding constants of the oligopeptide mixtures for estradiol from the competition curves (Fig. 1). The binding constants are shown in Table 1.

As all these results could be affected by contributions from residual estradiol, we performed a template and blank polymer-

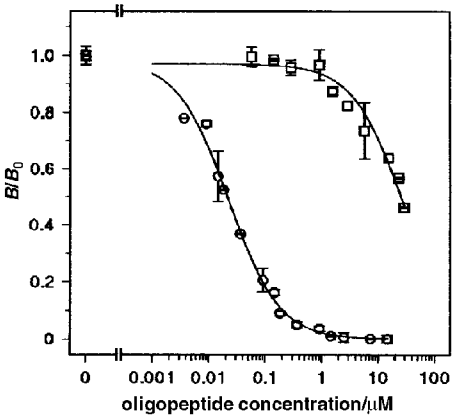

Fig. 1 Immunocompetition of branched mixtures polymerised for 1 hour. The plot of B/B_0, where B is the binding of the tritium labelled estradiol with the antibody in the presence of the oligopeptides and B_0 without the oligopeptides, *vs*. the oligopeptide concentration, gives competition curves from which the binding constant of the oligopeptides towards estradiol can be calculated. The branched template oligopeptides (○) and the branched blank oligopeptides (□) are shown.

Table 1 Binding constants of oligopeptide mixtures

Oligopeptide mixtures	Polymerisation time/h	Template K/M^{-1}	Blank K/M^{-1}
Branched	1	9.0×10^8	9.0×10^6
	3	1.2×10^9	1.5×10^7
	30	6.6×10^9	1.3×10^9
Linear	1	4.4×10^8	2.2×10^6
	3	5.8×10^8	2.8×10^6
	30	1.4×10^9	3.6×10^8

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isation in the presence of labelled estradiol.[10] After purification, the amount of bound estradiol was determined by this method, and only 3–4% of the template polypeptides contained bound estradiol. Control experiments were therefore carried out to investigate the effect of bound estradiol. Therefore, we corrected the mathematical equation, including a parameter that allowed us to take into account the presence of some residual estradiol. Fitting this last equation to the experimental data, we observed that the binding constants were essentially unchanged. So, the reported results are not affected by any contribution from residual estradiol, as the great excess of high affinity oligopeptides prevents the effective displacement of labelled estradiol from antibody binding sites by the residual template molecule.

To further verify the absence of artefacts in the evaluation of binding constants (due to the presence of residue template molecules), we performed some preliminary ultrafiltration experiments.[11] All values of binding constants obtained are about one order of magnitude lower than those obtained by competitive binding experiments, both for blank and template mixtures, but are affected by great imprecision. The reason for this is related to the partial adsorption of estradiol on the ultrafiltration membrane and also to the partial ultrafiltration of the oligopeptides, as determined in separate experiments with tritium labelled oligopeptides. The method is thus insensitive to residual estradiol but the partial ultrafiltration of oligopeptides leads to an underestimation of the binding. For these reasons we abandoned the ultrafiltration technique in favour of the competitive binding assay, which is simple to perform and gives better results.

In order to verify that there were no interactions between the rabbit anti-estradiol antibodies or the goat anti-rabbit γ-globulin antibodies and the oligopeptides, we checked the interactions of labelled oligopeptides (template and blank mixtures) with the immobilised antibodies.[12] In both cases we registered no significant binding to the solid phase. So, the inhibition of binding between labelled estradiol and its antibody is caused only by the binding properties of the oligopeptide mixtures that bind the steroid.

From the previously reported results, we note the very high affinity of the oligopeptides for the templating molecule. The difference between the binding constants of the template and blank mixtures shows the importance of the template effect on the amino acid mixtures and the efficacy of these systems as competitors of the antibodies themselves. For all mixtures the binding constants increase with the polymerisation time; this effect can be explained by the formation of more and more complex structures which are able to interact better with estradiol. The ratio between template and blank decreases when we increase the polymerisation time, probably because in the blank mixtures a longer reaction time increases the formation of more complex oligopeptide structures able to rearrange themselves around the steroid molecule to obtain a greater interaction. Instead, in the template mixtures this effect is less important as the binding properties are mainly due to the imprint.

An important parameter to consider is the specificity of binding. We performed an immunocompetition using the branched oligopeptides (1 h, 30 h) to evaluate the binding constant of this mixture for structural homologues, such as the steroids, testosterone and progesterone. The experimental procedure was similar to those used previously.[13] The results indicate that the oligopeptides do not have significant affinity for these molecules. For template branched oligopeptides (1 h) the binding constant for the testosterone is 1.2×10^5 M^{-1} *vs.* 9.0×10^8 M^{-1} for the estradiol, whereas for the blank ones K is 3.3×10^4 M^{-1} *vs.* 9.0×10^6 M^{-1}. The binding constants obtained for the branched oligopeptides polymerised for 30 h (template: 7.7×10^5 M^{-1}; blank: 4.8×10^6 M^{-1} for testosterone) confirm the hypothesis that longer reaction times can result in structures which can rearrange themselves to better match the steroid molecule without loss of selectivity (this remains unchanged), thus indicating that the imprinting effect is operative in all cases. For progesterone it is possible only to estimate an upper limit for the binding constant (1.0×10^4 M^{-1}). This is further evidence of the high affinity and high specificity of recognition toward the templating molecule. Moreover the template mixtures have selectivities similar not only to usual antisera, but also to the molecular imprinted polymers.[14]

Notes and references

1 C. Andreu, A. Galan, K. Kobiro, J. De Mendosa, T. K. Park, J. Rebek, A. Salmeron and N. Usman, *J. Am. Chem. Soc.*, 1994, **116**, 5501.
2 M. Yoshida, K. Uezu, M. Goto and S. Furusaki, *J. Appl. Polym. Sci.*, 1999, **73**, 1223.
3 M. Kempe, *Anal. Chem.*, 1996, **68**, 1948.
4 Y. Liao, W. Wang and B. Wang, *Bioorg. Chem.*, 1998, **26**(6), 309.
5 T. Takeuchi, D. Fukuma and J. Matsui, *Anal. Chem.*, 1999, **71**, 285.
6 The polymerisation was performed in mixed aqueous/organic media (carbonate buffer 0.2 M pH 9/DMF, 1 + 1) using carbodiimide as a condensing agent. The mixtures were shaken according to the different reaction times at room temperature in the dark. Then the oligopeptide mixtures were filtered on 0.45 μm sintered glass.
7 We washed the resin with carbonate buffer (20 mM, pH 8.5) until the absorbance had returned to the baseline. After reversing the direction of the flow, the elution was performed by running a phosphate buffer (50 mM phosphate, 1 M NaCl, 1 mM EDTA, pH 7).
8 K. Stulik, V. Pacakova, J. Suchankova and H. A. Claessens, *Anal. Chim. Acta*, 1997, **352**, 1.
9 G. Giraudi, I. Rosso, C. Baggiani and C. Giovannoli, *Anal. Chim. Acta*, 1999, **381**, 133.
10 Labelled estradiol (2.8×10^{-2} μM) was added to the estradiol and the amino acid mixtures before polymerisation. During the purification, we followed the absorbance of the oligopeptides with a spectrophotometric detector and the eluate was fractionated every 5 cm^3 to check the signal of the labelled estradiol.
11 We added the oligopeptides at increasing concentrations to a constant amount of labelled estradiol, and after centrifugation in an Amicon MPS-1 device, equipped with YM1 membranes (nominal molecular weight cut-off of 1000 Da), we measured the ultrafiltered estradiol in the filtrate. The fraction of estradiol in the retentate plotted *vs.* the oligopeptide concentration allowed us to estimate the value of binding constants.
12 For the synthesis of the labelled oligopeptides we added volumes of labelled Ser, Leu, Gly, Glu (each amino acid was 1.0 mCi cm^{-3}) to the amino acid solution, and then we performed the polymerisation as previously described. These quantities were proportional to the percentage of the corresponding amino acid in the polymerisation mixture and assured a significant measure of radioactivity. The small volumes added can be considered negligible in comparison to the volume of the whole polymerisation mixture.
13 We used antibodies directly against testosterone and tritium labelled testosterone for the affinity measurement of this steroid, and anti-progesterone antibodies and tritium labelled progesterone for the determination of the affinity for this one.
14 O. Ramstroem and K. Mosbach, *Chem. Biol.*, 1996, **3**, 471.