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Ferrocene-containing nucleic acids. Synthesis and electrochemical properties

T S Zatsepin, S Yu Andreev, T Hianik, T S Oretskaya

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Abstract. The published data on the synthesis of ferrocene-containing nucleic acid fragments are generalised. The known methods are systematised and their advantages and disadvantages are discussed. The use of nucleoside, nucleotide and oligonucleotide conjugates with ferrocene in the design of systems for electrochemical detection of nucleic acids is considered. The bibliography includes 121 references.

I. Introduction

Studies of nucleic acids (NA) and their complexes with proteins require the development of highly sensitive procedures for their identification and quantitation in analysed samples. The use of spectrophotometric, autoradiographic and electrochemical techniques for the analysis of nucleic acids envisages usually incorporation of appropriate reporter groups. Incorporation of reporter groups favours higher selectivity of analysis and allows detection of low concentrations of NA with a minimum effect on their structures and functions.

Labelling of NA with radioactive isotopes, ^{32}P in particular (see Ref. 1), has become a very popular procedure. Phosphorus isotopes are incorporated by enzymatic methods using labelled nucleoside 5′-triphosphates or nucleoside 3′,5′-diphosphates. The efficiency of this reaction is very high for both short-chain and extended NA. However, manipulations with radioactive isotopes demand certain precautions. Other limitations of the method include short half-lives of ^{32}P, ^{35}S and ^{125}I and radiolysis of labelled NA.

The use of fluorescent labels has received the widest acceptance for the detection of nucleic acids.[2,3] 5′-Triphosphates of nucleosides, 2′-deoxynucleosides and 2′,3′-dideoxynucleosides containing various fluorescent labels are commercially available at present. The use of nucleoside triphosphate conjugates with biotin in enzymatic synthesis of NA enables detection and selective isolation of target oligonucleotides due to the formation of stable biotin – avidin or biotin – streptavidin complexes immobilised on a polymeric substrate.[4] Detection of nucleic acids by mass spectrometry [MALDI-MS (see Ref. 5) and ESI-MS (see Ref. 6)] is also being developed at a racing pace.

Electrochemical detection holds a tremendous potential in the analysis of nucleic acids. High sensitivity, low cost, the possibility of performing express analysis and, especially, simplicity of miniaturisation make electrochemical detection a promising tool in the design of DNA chips,[7] in HPLC and capillary electrophoresis of NA.[8]

Synthesis of modified nucleosides, nucleotides and oligonucleotides containing various metal complexes is an approach to the design of electrochemical NA sensors.[9] Conjugates of modified oligonucleotides with metal ions can also be used as chemical nucleases [10,11] and in the studies of electron transfer in DNA.[12–14] There are several methods for the synthesis of such compounds: (1) conjugation of a metal complex and an oligonucleotide containing a reactive group at the 5′- or 3′-end;[15,16] (2) addition of metal ions to an oligonucleotide bonded to a chelating group;[17,18] (3) synthesis of a nucleoside phosphoramidite derivative containing a metal complex [19,20] and its subsequent incorporation into the oligonucleotide chain by automatic solid-phase synthesis. A recently published review [21] is devoted to the synthesis of oligonucleotide complexes with platinum-group metals.

The present review deals with the methods of the synthesis and applications of ferrocene-containing nucleosides, nucleoside-5′-triphosphates and oligonucleotides. Nucleosides can be incorporated into NA *via* automated oligonucleotide synthesis and nucleoside-5′-triphosphates enzymatically respectively. Owing to their small size, high stability of oxidised and reduced forms, reversibility and high rates of redox reactions and accessibility, ferrocene derivatives represent promising starting compounds for the synthesis of metal-containing oligonucleotides.[22]

Immobilisation of ferrocene-containing oligonucleotides on electrodes permits determination of the primary structure of NA † by hybridisation. An alternative approach to the study of nucleotide sequences entails the synthesis of ferrocene conjugates with various molecules capable of selective and effective binding to NA.[24] In some cases, ferrocene conjugates with intercalators are used for this purpose.[25–27] However, the use of ferrocene-containing oligonucleotides ensures the highest specificity of binding

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† It is known [23] that peptides containing a ferrocene moiety at the *N*-end are used for the studies of spatial structures by electrochemical methods and X-ray diffraction analysis. Unfortunately, no such studies of ferrocene-containing NA have been carried out so far.

to NA targets in comparison with other ferrocene derivatives suggested.

Electrochemical detection of NA on the electrode surface is a promising approach to the study of single nucleotide polymorphism (SNP), the most common type of genome mutations affecting one out of one thousand nucleotide units. It has been shown [28] that these mutations are responsible for certain genetic diseases. Direct sequencing studies have demonstrated [29] that the human genome contains about 1 420 000 SNP. Other popular procedures for recognition of SNP include the polymerase chain reaction (PCR) with nucleoside triphosphates bearing fluorescent labels [30] and the use of DNA chips.[31]

In addition, studies of SNP may include an analysis of single- [32] and double-stranded [33] conformational polymorphism and hybridisation in solution followed by gel electrophoresis with denaturing gradient [34] or temperature gradient.‡

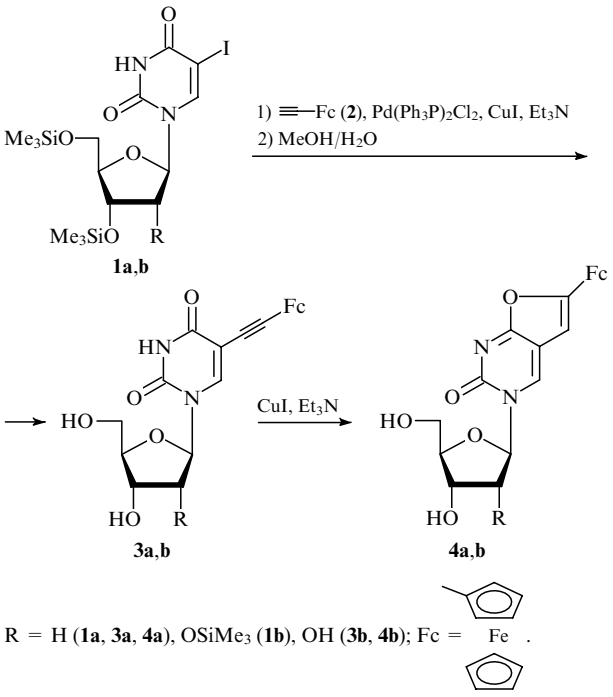
Thus, the methods for the synthesis of ferrocene-containing oligonucleotides and their applications described in this review present interest for specialists in the chemistry of nucleic acids and those engaged in the diagnostics of genetic diseases.

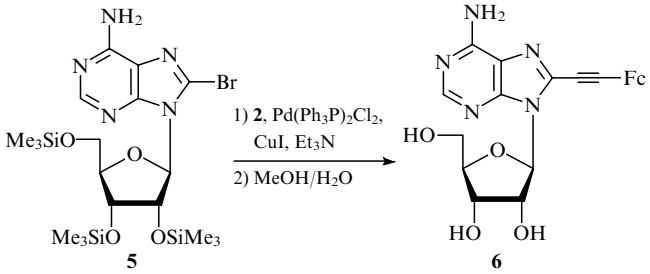
II. Synthesis of ferrocene-containing nucleic acid fragments

1. Synthesis of nucleic acid fragments containing ferrocene in a heterocyclic base

The first paper devoted to the synthesis of ferrocene-containing heterocyclic bases and nucleosides was published in 1991.[35] It described several synthetic strategies.

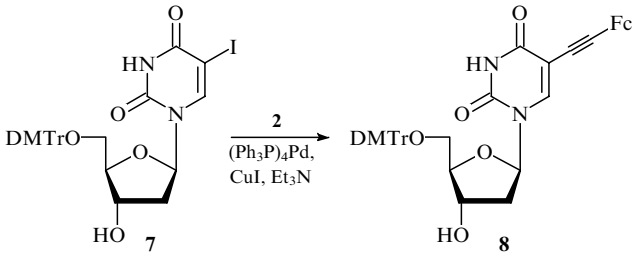
The first of them is based on the reaction of protected 5-iodo-2′-deoxyuridine (**1a**) or 5-iodouridine (**1b**) with ethynylferrocene **2** under conditions of the Sonogashira reaction,[36,37] *viz*., stirring of the reactants in triethylamine in the presence of catalytic amounts of bis(triphenylphosphine)palladium dichloride and copper(I) iodide at 40 °C. The yields of the pyrimidine derivatives **3a**,**b** after removal of trimethylsilyl protective groups were as low as 10% – 15%, since under the conditions used these compounds are partly converted into ferrocenylfuropyrimidones **4a**,**b**. The analogous reaction of 8-bromoadenosine **5** yielded 70% of the ferrocenyl derivative **6**.[35]





It has been shown [38] that the 5-(ferrocenylethynyl)uridine derivatives **3a**,**b** being incorporated into NA were converted into ferrocenylfuropyrimidones **4a**,**b** upon standard postsynthetic treatment of oligonucleotides with aqueous ammonia. The presence of furopyrimidone in the oligonucleotide chain reduces thermal stability of a DNA duplex.

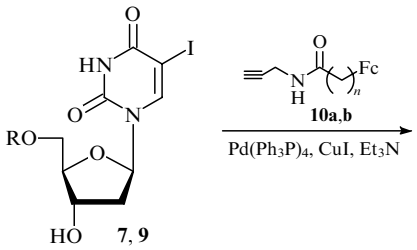
Later, Houlton *et al*.[39] used another palladium catalyst, *viz*., tetrakis(triphenylphosphine)palladium, and reduced the reaction temperature to room temperature. Such a change in the experimental conditions and the presence of a 5′-dimethoxytrityl protective group, which enhances the solubility of the starting nucleoside **7,** increase the yield of the substitution product **8** to 75%.



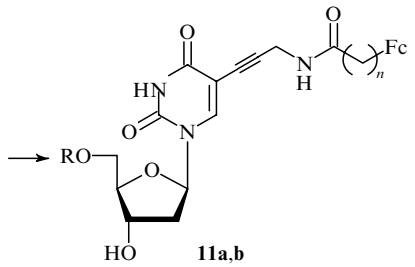
DMTr = $(4\text{-MeOC}_6H_4)_2PhC$.

Prior to the incorporation of the nucleoside **8** into the oligonucleotide chain, this was converted into a phosphoramidite derivative. The use of acetyl or phenoxyacetyl groups for the protection of exocyclic amino groups of heterocyclic bases in the oligonucleotide synthesis allowed postsynthetic deprotection of the target oligomers under milder conditions, *viz*., with 50 mM potassium carbonate in methanol instead of concentrated ammonia, which reduced the risk of cyclisation reactions. However, subsequent hydrolysis of the modified oligonucleotides with snake venom phosphodiesterase and alkaline phosphatase revealed that about 50% of ferrocene-containing nucleosides existed as ferrocenylfuropyrimidones. Storage of oligonucleotides in solution causes gradual oxidation of ferrocene with atmospheric oxygen and results in the formation of a ferrocenium cation as demonstrated by reversed-phase HPLC. Therefore, dithiothreitol was added to the solution to stabilise modified uridine residues.[39]

At present, the Sonogashira reaction is still the most popular procedure for the introduction of a ferrocenyl unit into a heterocyclic base.[38–43] Thus compounds **11a**,**b** were prepared starting from 5-iodo-2′-deoxyuridine **9** or its 5′-dimethoxytrityl derivative **7** and alkynyl derivatives of ferrocene **10a**,**b**. The reaction of compound **10a** with protected 8-bromo-2′-deoxyadenosine **12** yielded a modified nucleoside **13**.

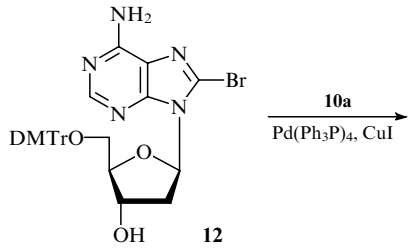


‡ R M Wartell, S Hosseini, S Powell, J Zhu *J. Chromatogr. A* **806** 169 (1998).

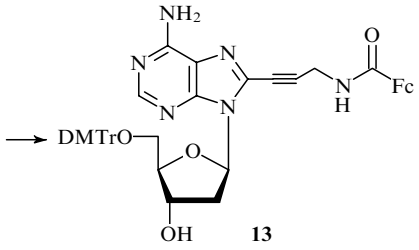


11a,**b**

R = DMTr (**7**), H (**9**); n = 0 (**a**), 2 (**b**).

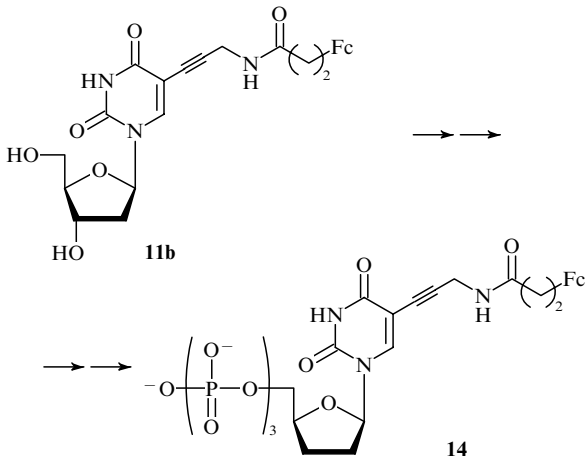


12



13

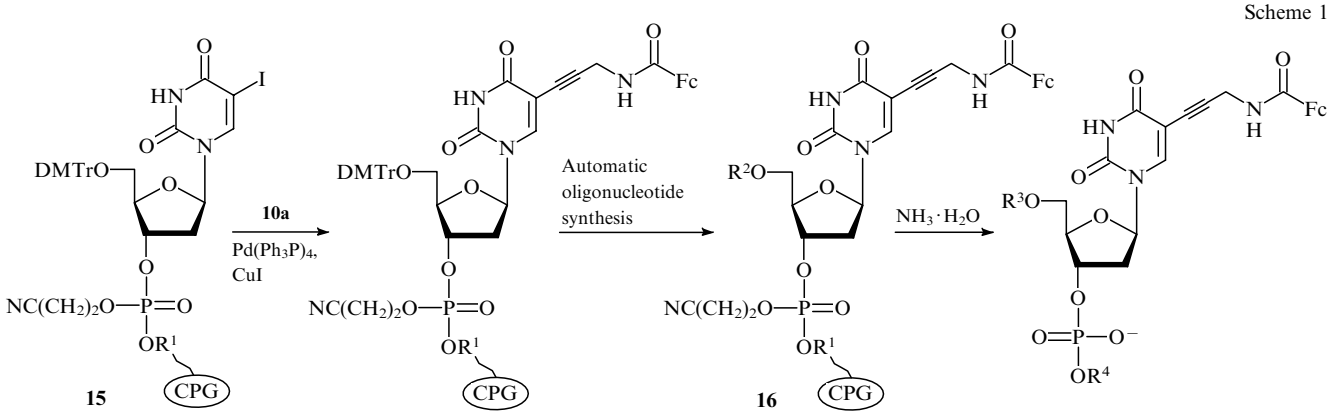
Modified 2′-deoxyuridine **11b** bearing a ferrocene derivative at C(5) was converted into 2′,3′-dideoxy-5′-triphosphate **14** and attached to the 3′-end of an oligonucleotide using terminal deoxynucleotidyl transferase.[42] Compound **14** and its analogues can be used for DNA sequencing according to Sanger[44] with electrochemical detection.

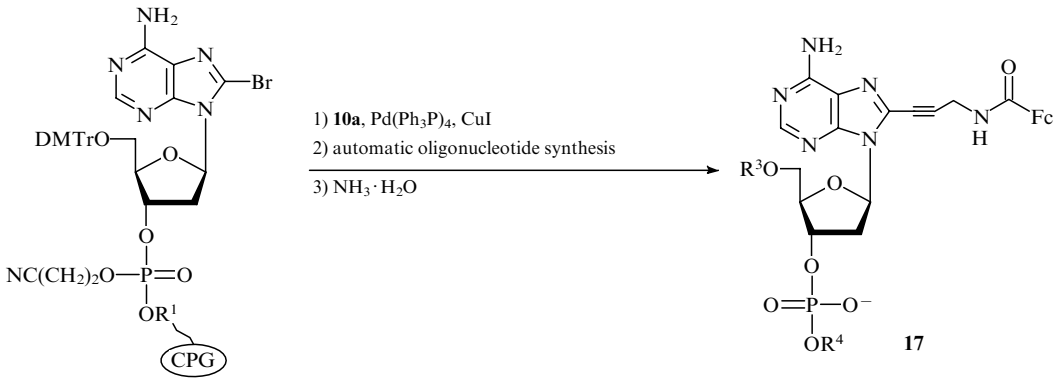


11b **14**

Grinstaff *et al.*[40,41,45] have developed an original procedure for the incorporation of ferrocenyl derivatives into a growing oligonucleotide chain. This involved coupling of the propynyl ferrocene derivative **10a** with a modified oligomer **15** obtained by attachment of protected 5-iodo-2′-deoxyuridine **7** to the 5′-end of the protected immobilised oligonucleotide in the presence of a palladium catalyst and copper(I) iodide after which the oligonucleotide synthesis was continued. In the last step, the modified oligomer **16** was deprotected with concentrated aqueous ammonia (Scheme 1). Oligonucleotides **17** containing a modified deoxyadenosine residue were prepared in a similar way. The Sonogashira reaction is extremely sensitive to numerous factors, particularly to the presence of trace amounts of primary amines and water; therefore, the yields of the oligonucleotides were 20% – 50%. It is of note that the yield of the target product **16** decreased to 8% when coupling of the ferrocene derivative **10a** was carried out after termination of the oligonucleotide synthesis. This can be attributed to steric hindrances created by the 5′-terminal fragment of the immobilised oligonucleotide. The yields of

Scheme 1

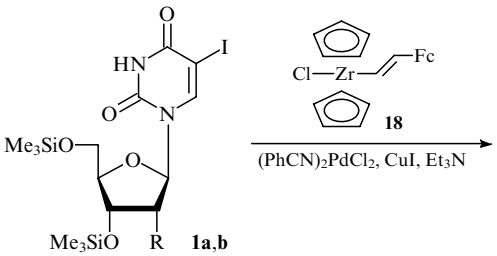


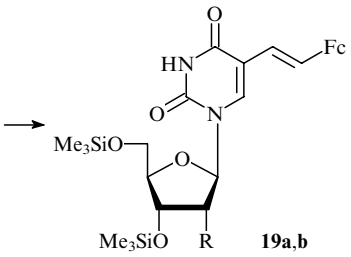


R^1, R^2 are fragments of a protected oligonucleotide chain; R^3, R^4 are fragments of an oligonucleotide chain; CPG, controlled pore glass.

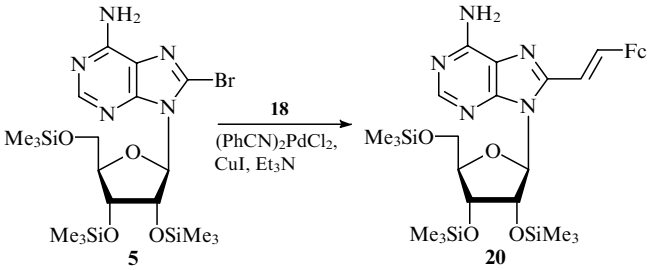
the modified oligomers after incorporation of the ferrocene-containing nucleosides **11a** and **13** into the oligonucleotide chain were 10% – 15%. The presence of a ferrocenyl-2′-deoxyadenosine derivative in the oligonucleotide chain induces greater destabilisation of a DNA duplex in comparison with its 2′-deoxyuridine analogue.

The second approach [35] to the synthesis of NA fragments containing a ferrocene residue in the heterocyclic base includes condensation of the halogenonucleosides **1a**,**b** and **5** with the organometallic vinylferrocene derivative **18**. The yields of the products **19a**,**b** and **20** were 15% – 20%, which is significantly lower in comparison with the Sonogashira synthesis.

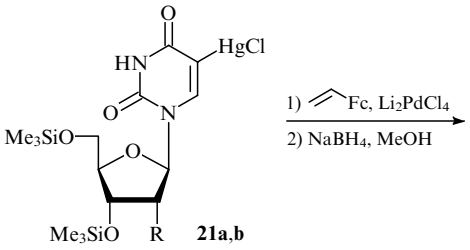




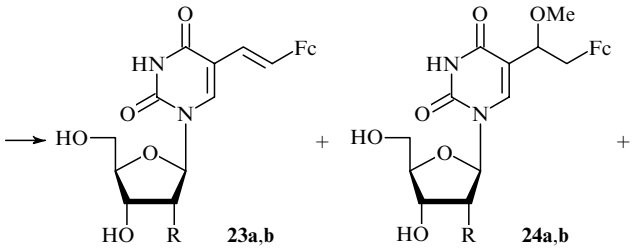
R = H (**a**), $OSiMe_3$ (**b**).

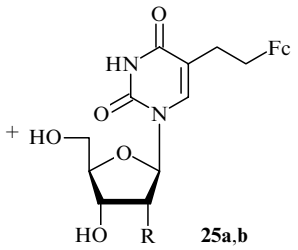


The third variant of the synthesis of NA fragments modified with ferrocene at the heterocyclic base [35] consists of cross-coupling of 5-chloromercurioorganic derivatives of uridine **21a**,**b** and cytosine **22a** – **c** with vinylferrocene in the presence of lithium tetrachloropalladate and subsequent treatment with sodium borohydride in methanol. This represents a modification of the Heck reaction.[46,47] Methanol present in the reaction mixture interacts with the exocyclic double bond of the modified nucleoside, whereas sodium borohydride partly reduces it. Thus, the reaction of the uridine derivatives **21a**,**b** affords, along with the cross-coupling products **23a**,**b**, compounds **24a**,**b** and **25a**,**b** as well. Reaction of the cytidine derivatives **22a**,**b** with vinylferrocene gave the corresponding ferrocene-containing nucleosides **26** – **28**. In both cases, the total yields of the modified nucleosides were

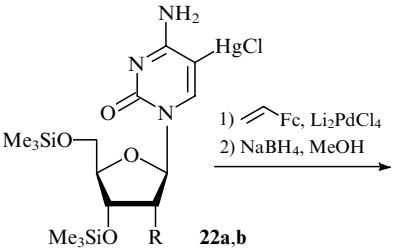


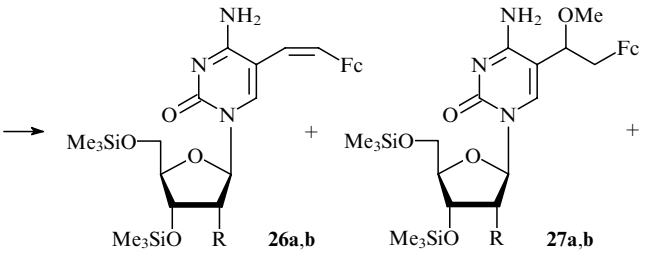
15% – 30%. The conditions favouring the predominant formation of one of the reaction products were selected.[35]

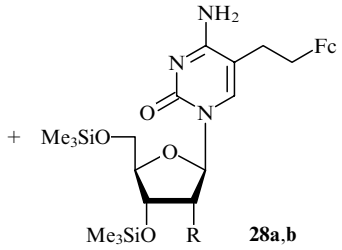




21: R = H (**a**), $OSiMe_3$ (**b**); **23** – **25**: R = H (**a**), OH (**b**).



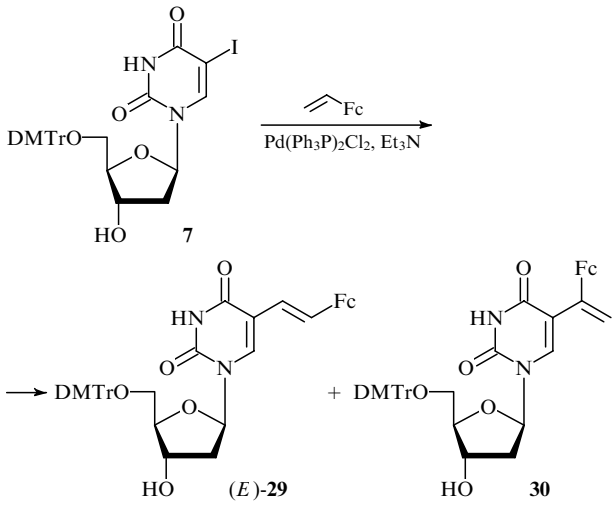




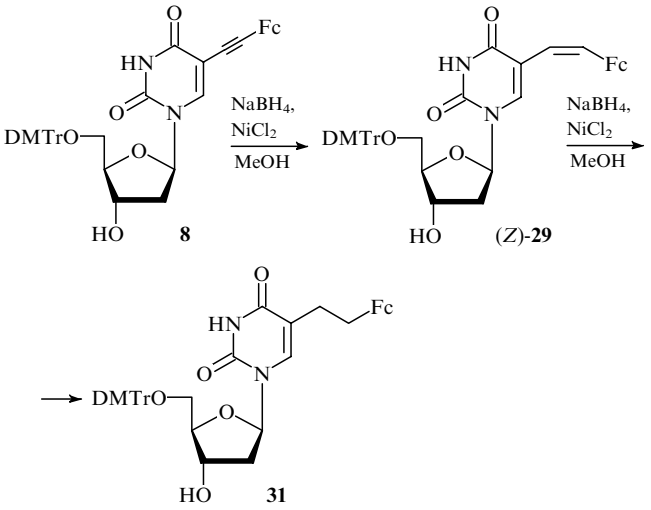
22: R = H (**a**), $OSiMe_3$ (**b**); **26** – **28**: R = H (**a**), OH (**b**).

The cytotoxic activities of deprotected 5-ferrocenylvinyl-2′-deoxyuridine **23a** and 5-ferrocenylvinyluridine **23b** were studied in L1210 cells *in vitro*.[35] The lethal dose causing death of 50% of cells (LD_{50}) was by several orders of magnitude lower than that of the traditional antibiotic adriamycin and commensurable with that of ferrocenylmethanol.

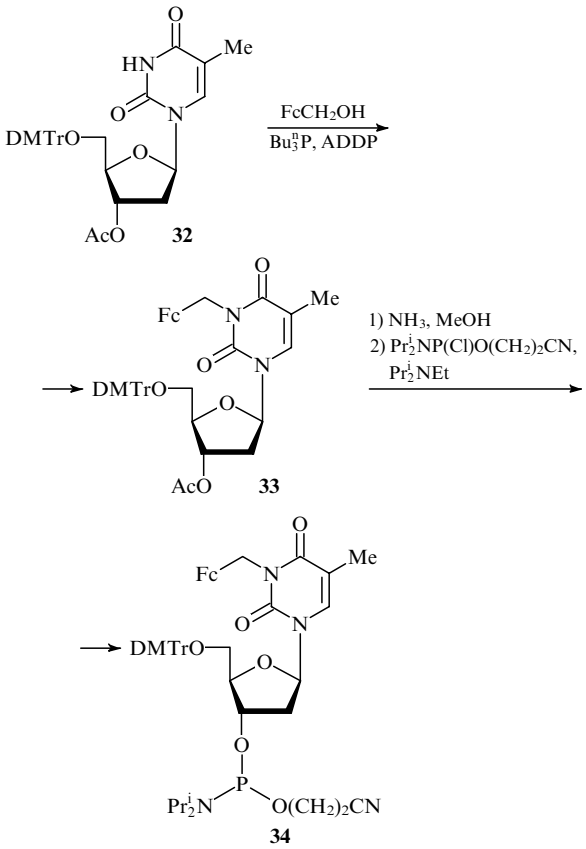
The Heck alkenylation of protected 5-iodo-2′-deoxyuridine **7** with vinylferrocene resulted in the formation of two products, (*E*)-**29** and **30** (3 : 1), in a total yield of 60%.[39,43] An attempt to separate these compounds by TLC and HPLC failed; however, after deprotection of the 5′-hydroxy group (*E*)-5-ferrocenyl vinyl-2′-deoxyuridine was isolated by recrystallisation.



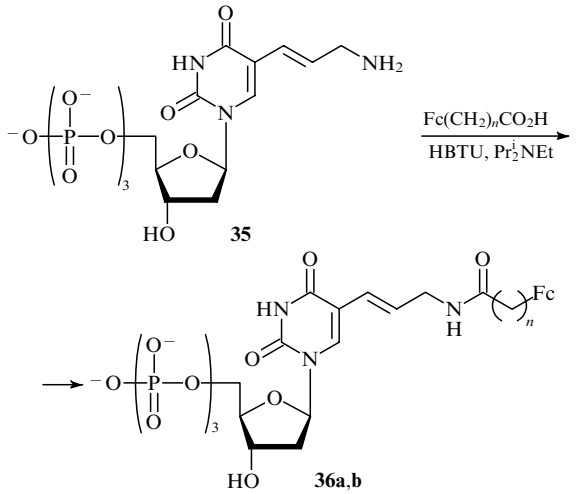
The *Z*-isomer of protected 5-ferrocenylvinyl-2′-deoxyuridine **29** was obtained by Houlton *et al.*[39,43] by reduction of protected 5-ferrocenylethynyl-2′-deoxyuridine **8** with sodium borohydride in the presence of nickel chloride (yield 30%). Further reduction of compound (*Z*)-**29** under the same conditions afforded 5′-dimethoxytrityl-5-ferrocenylethyl-2′-deoxyuridine **31** in 25% yield.



A method[48] for the introduction of a ferrocenyl group at the N(3) atom of protected thymidine **32** by Mitsunobu reaction[49] of ferrocenylmethanol in the presence of tributylphosphine and diethyl azodicarboxylate (DEAD) in THF at room temperature has been proposed. The modified nucleoside **33** was further converted into the phosphoramidite derivative **34** employed for the preparation of ferrocene-containing oligonucleotides. Stacking interactions in such oligonucleotides are strongly violated as can be judged from CD spectroscopic data. As a consequence, duplexes of these oligonucleotides with DNA and RNA are less stable in comparison with natural ones, which makes this modification unacceptable for the design of NA detectors. Incorporation of a phosphoramidite derivative of the modified nucleoside **34** into the oligonucleotides increased the thermal stability of triplexes with double-helical DNA by 1–6 °C; however, their structures differed from the classical one (CD spectroscopic data). An attempt to explain the stabilisation phenomenon failed.[48]

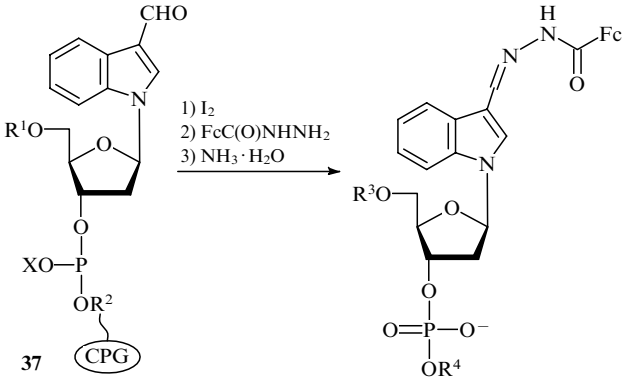


A method for the synthesis of a modified ferrocene-containing nucleoside triphosphate has been proposed.[50] 5-(3-Aminoprop-1-enyl)-2′-deoxyuridine triphosphate **35** was acylated with ferrocenecarboxylic and ferrocenylacetic acids in the presence of a condensation reagent, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). The yields of compounds **36a**,**b** were only 30 and 8%, respectively. Nevertheless, this approach seems to be promising by virtue of its simplicity. Interestingly, the ferrocenecarboxylic acid derivative **36a** is a substrate of DNA polymerase, whereas its structural analogue, the ferrocenylacetic acid derivative **36b**, terminates the enzymatic reaction of elongation of the oligonucleotide chain.



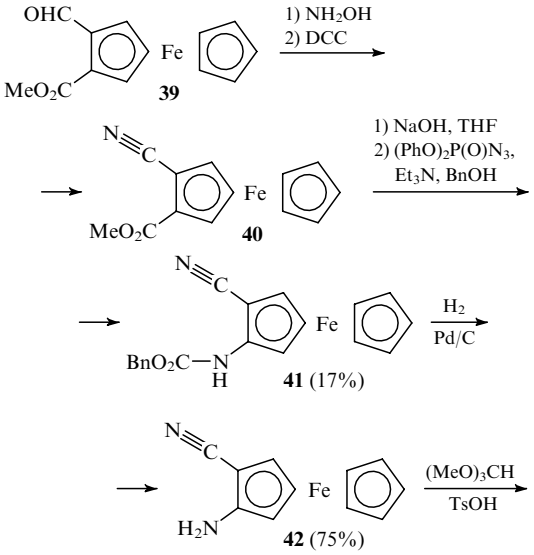
n = 0 (**a**), 1 (**b**).

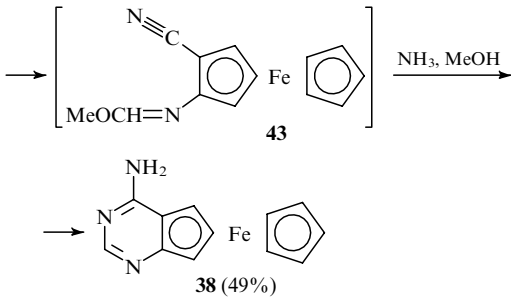
It was recommended [51] to attach ferrocenecarbohydrazide to the aldehyde group of a nonnative heterocyclic base of the oligonucleotide **37** immobilised on a polymeric support. However, the stabilities of the free aldehyde group in compound **37** with respect to oxidation with iodine under conditions of a standard phosphoramidite oligonucleotide synthesis and of the hydrazone formed in the standard postsynthetic treatment with aqueous ammonia are doubtful.



X = $NC(CH_2)_2$; R^1, R^2 are fragments of a protected oligonucleotide chain; R^3, R^4 are fragments of an oligonucleotide chain.

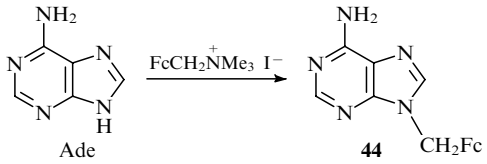
A multistep synthesis of an adenine analogue **38** from a ferrocene derivative **39** has been described.[52] The aldehyde group in the latter was converted into a cyano group in two steps, *viz*., by reaction with hydroxylamine to give an oxime and dehydration with dicyclohexylcarbodiimide in boiling benzene. The ester group in nitrile **40** was hydrolysed to a carboxy group using NaOH in THF. The reaction of the acid formed with diphenyl phosphoryl azide in the presence of triethylamine and benzyl alcohol was accompanied by Curtius rearrangement and gave compound **41** as the intermediate product; catalytic hydrogenolysis of the latter yielded aminonitrile **42**. Its one-pot reaction with triethyl orthoformate in the presence of *p*-toluenesulfonic acid and treatment of the imidate **43** with methanolic ammonia resulted in compound **38**. The structure of the latter was confirmed by ^1H, ^{13}C NMR and IR spectroscopy and mass spectrometry. Further attempts will be aimed at the synthesis of the corresponding adenosine analogue which can be introduced into the oligonucleotide chain and used for the study of electron transfer in DNA duplexes. Most probably, the presence of such a modification within the oligonucleotide chain will strongly impede stacking interactions between 'stacked' heterocyclic bases.



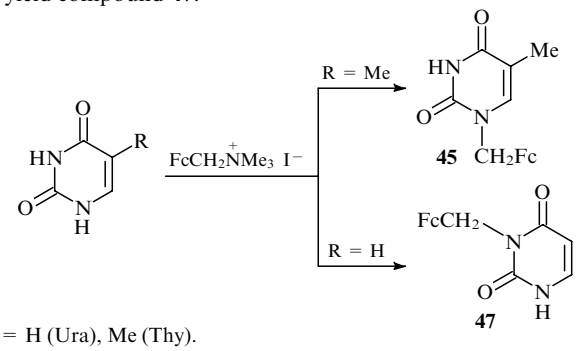


DCC = cyclo-$C_6H_{11}-N=C=N-C_6H_{11}$-cyclo.

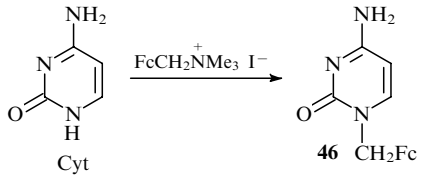
The synthesis of purine and pyrimidine heterocyclic bases containing a ferrocene moiety has been described.[53, 54] 9-Ferrocenylmethyladenine (**44**) was prepared by alkylation of adenine (Ade) with (ferrocenylmethyl) trimethylammonium iodide.[53]



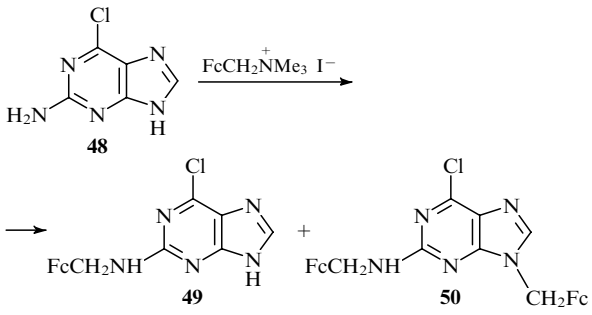
The use of this alkylating reagent enables preparation of N(1) thymine (Thy) and cytosine (Cyt) derivatives **45** and **46** as the main products, whereas uracil (Ura) is preferentially alkylated at N(3) to yield compound **47**.[54]

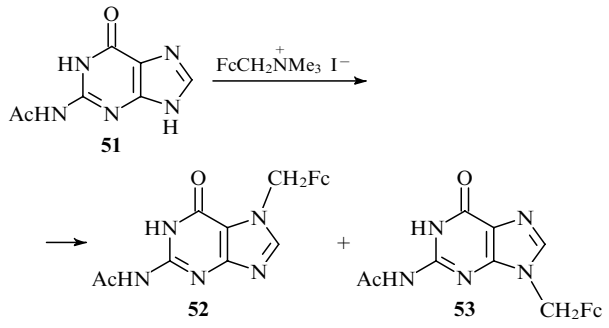


R = H (Ura), Me (Thy).

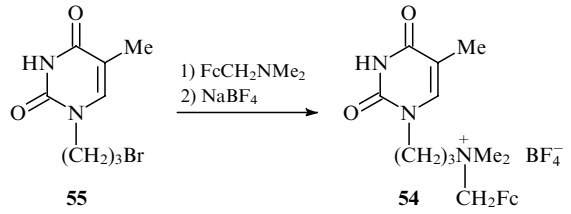


N(2)-mono- and N(2),N(9)-dialkyl derivatives **49** and **50** were isolated from 2-amino-6-chloropurine **48** as the guanine precursor, whereas the alkylation of 2-acetylguanine **51** occurred at N(7) and N(9) and afforded the products **52** and **53**. The structures of the synthesised compounds were established by X-ray diffraction analysis.

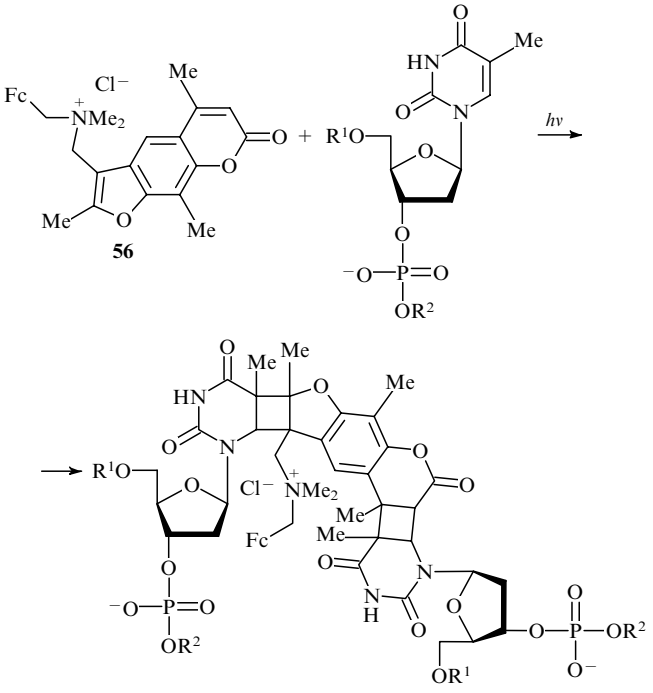




1-[3-(*N*,*N*-Dimethyl-*N*-ferrocenylmethyl)ammoniopropyl]thymine **54** was prepared by a reaction of 1-(3-bromopropyl)thymine **55** with (ferrocenylmethyl)dimethylamine and isolated in the form of a tetrafluoroborate.[53]



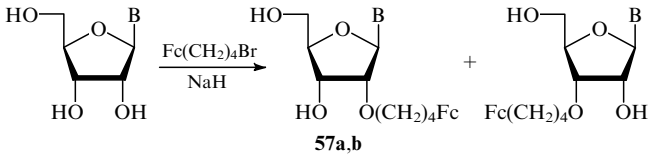
A method for the introduction of ferrocene residues into oligonucleotides based on a photochemical reaction of a ferrocenyl derivative of psoralene with thymine residues within double-stranded DNA has been developed.[55] To this end, *N*,*N*-dimethylaminomethylferrocene was alkylated with chloromethyl psoralene and the resulting compound **56** reacted with DNA in a buffer solution upon irradiation with the light of a mercury lamp (λ = 365 nm) for 10 min. This resulted in an efficient formation of ferrocene-containing conjugates of double-stranded DNA. The presence of a ferrocene residue was confirmed by cyclic voltammetry. This method enables electrochemical identification of double-stranded DNA, since the ferrocenyl–psoralene derivative reacts exclusively with the DNA duplex.



R^1, R^2 are fragments of an oligonucleotide chain.

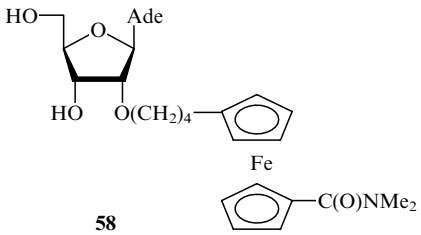
2. Synthesis of nucleosides and oligonucleotides containing ferrocene in the 2′-position of the carbohydrate moiety

There are several methods for the synthesis of 2′-*O*-alkylnucleosides[56] among which alkylation of free nucleosides with alkyl halides in the presence of sodium hydride is one of the most widespread and efficient procedures. In this case, the selectivity of the reaction involving the 2′-hydroxy group is explained by its higher acidity in comparison with 3′- and, especially, 5′-hydroxy groups. This method was used [57, 58] for the synthesis of nucleosides containing ferrocenyl units in the 2′-position of the carbohydrate moiety.§ The yields of the alkylation products of adenosine **57a** and cytidine **57b** were 40 (isomer ratio 2′ : 3′ = 2.7 : 1) and 76% (isomer ratio 2′ : 3′ = 1.8 : 1), respectively.



B = Ade (**a**), Cyt (**b**).

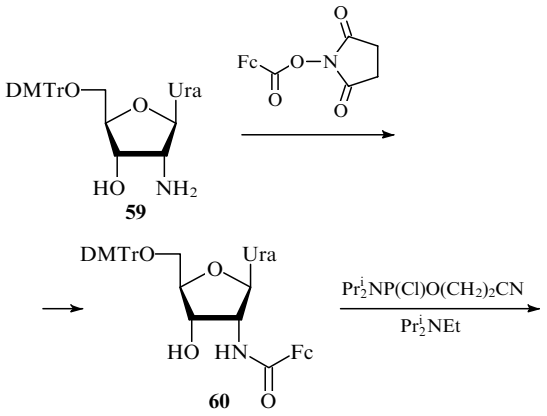
Compound **58** containing a dimethylcarbamoylferrocene residue in the 2′-position was synthesised in a similar way; it possessed a redox potential different from that of ferrocene.



Nucleosides **57a**,**b** and **58** were converted into phosphoramidite derivatives that were employed in the automatic oligonucleotide synthesis. Incorporation of the modified nucleosides into the oligonucleotide chain did not change the thermal stability of the DNA duplexes, which is typical for 2′-*O*-alkyl nucleosides.[60]

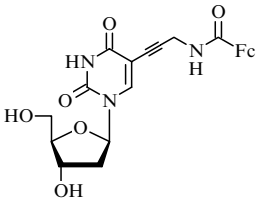
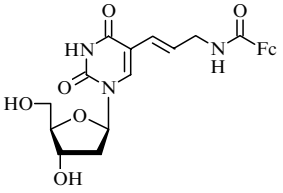
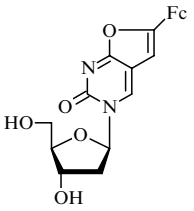
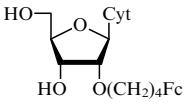
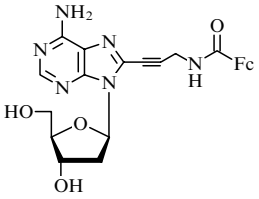
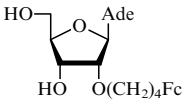
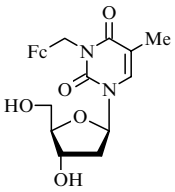
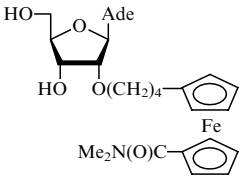
The data on the thermal stabilities of ferrocene-containing DNA duplexes are listed in Table 1.

Acylation of 5′-protected 2′-amino-2′-deoxyuridine **59** with *N*-hydroxysuccinimide ester of ferrocenecarboxylic acid is patented.[61–63] Subsequent phosphitylation of nucleoside **60** occurred at a low rate and resulted in compound **61** in a low yield (30%), which is due to the presence of a bulky substituent in the 2′-position of the carbohydrate fragment.



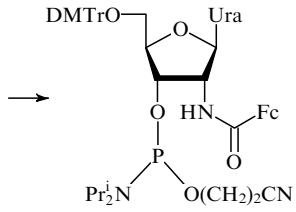
§ The first report on the use of oligonucleotides containing ferrocene in the 2′-position of the carbohydrate fragment was published three years earlier. However, no detailed description of this synthesis was given.[59]

Table 1. Changes in thermal stability (ΔT_m) of DNA duplexes upon incorporation of ferrocene-containing nucleosides into the oligonucleotide chain.

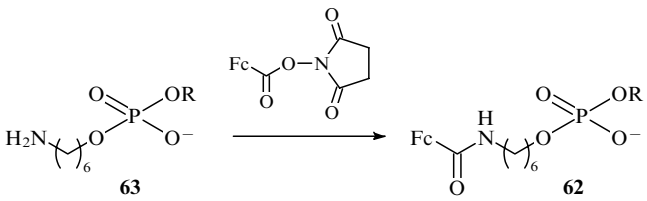
Modified nucleoside	ΔT_m /°C (see [a])	Conditions [b]	Ref.	Modified nucleoside	ΔT_m /°C (see [a])	Conditions [b]	Ref.
	−(1.2–2.6)	5 mM NaH_2PO_4, 50 mM NaCl, pH 7.0	41		−0.8	300 mM KH_2PO_4, pH 7.0 (see [c])	50
	−(5.5–5.7)	15 mM sodium citrate, 150 mM NaCl, pH 7.0	38		−0.4 to +0.4	15 mM sodium citrate, 150 mM NaCl, pH 7.0	57
	−(7.6–9)	5 mM NaH_2PO_4, 50 mM NaCl, pH 7.0	41		0.1	the same	57, 58
	−(0.4–7.7) (near the 5′-end) −(4.7–14.1) (inside the chain)	10 mM NaH_2PO_4, 100 mM NaCl, pH 7.0	48		−0.4	"	58

[a] $\Delta T_m = \dfrac{T_m \text{ (modified DNA duplex)} - T_m \text{ (natural DNA duplex)}}{\text{number of modifications per DNA duplex}}$

[b] Concentration of the DNA duplex, 2 μmol litre^{-1}. [c] Concentration of the DNA duplex, 0.6 μmol litre^{-1}.



61



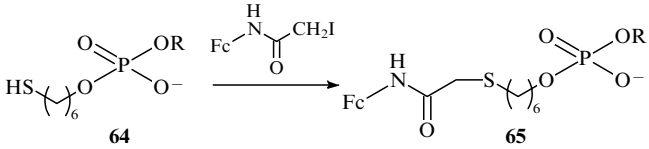
63 **62**

R is an oligonucleotide.

3. Synthesis of oligonucleotides containing ferrocene at the 5′- or 3′-termini

For the first time, the ferrocene-containing oligonucleotide **62** was prepared by acylation of the 5′-terminal amino group of compound **63** by *N*-hydroxysuccinimide ester of ferrocenecarboxylic acid [64–66] containing a ^{57}Fe isotope. Later, this approach was used many times,[67–71] *e.g.*, for the synthesis of an analogous derivative of ferrocenylacetic acid.[72] Incorporation of the modified compound into the oligonucleotide structure significantly increased the stability of the triplex with double-helical DNA (ΔT_m = 2.5–2.6 °C).

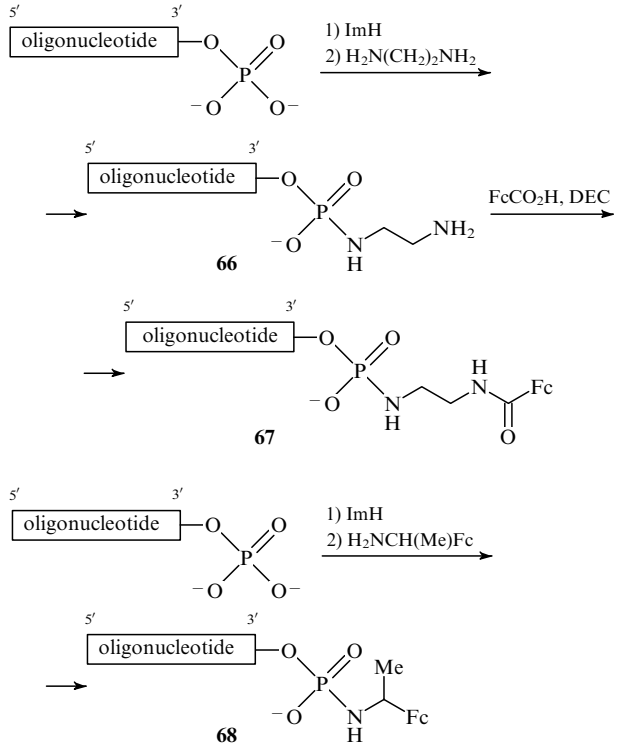
Recently, it has been proposed [73] to use iodoacetamidoferrocene, which interacts selectively with thiols, for modification of biomolecules. The specificity of incorporation of this reagent was demonstrated using the 5′-thiol-containing oligonucleotide **64** as an example.



64 **65**

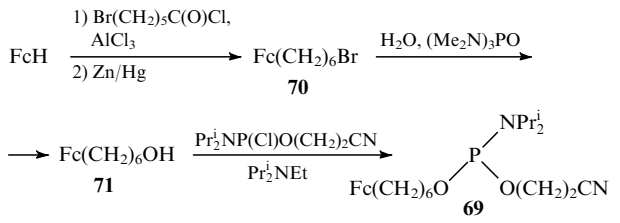
R is an oligonucleotide.

The introduction of a ferrocenyl residue at the 3′-end of the oligonucleotide was carried out using postsynthetic acylation of the 3′-terminal amino group of the oligonucleotide **66** with ferrocenecarboxylic acid in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC) [74] or the addition of 1-aminoethylferrocene to the activated 3′-phosphate group.[75]

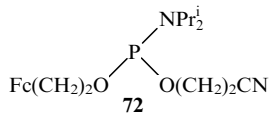


ImH is imidazole.

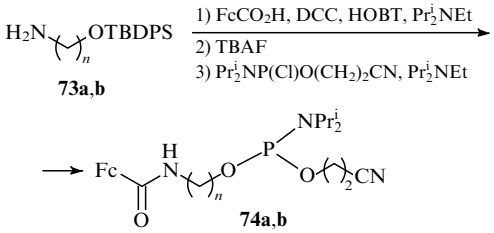
Letsinger *et al.*[76] recommended the use of the phosphoramidite derivative **69** for the synthesis of ferrocene-containing oligonucleotides. This approach is widely used for incorporation of such markers as biotin, acridine, fluorescein, *etc.* into oligonucleotides. 6-Bromohexylferrocene **70** was synthesised by the Friedel–Crafts acylation of ferrocene with 6-bromohexanoyl chloride with subsequent reduction of the carbonyl group with zinc amalgam. The bromo derivative **70** was converted into alcohol **71** and then into the phosphoramidite **69**.

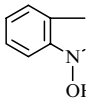


Synthesis of an analogous phosphoramidite **72** and its application for the synthesis of modified oligonucleotides have been described.[57]

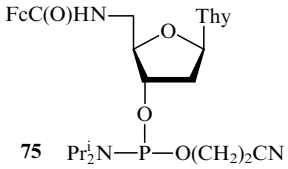


The synthesis of a series of phosphoramidite derivatives including nucleosides entails attachment of a ferrocene residue to the 5′-end of the oligonucleotide.[77] This is achieved by acylation of amino groups of compounds **73a**,**b** with ferrocenecarboxylic acid in the presence of a condensation reagent, *viz.*, dicyclohexylcarbodiimide. After removal of a *tert*-butyldiisopropylsilyl protective group by treatment with tetrabutylammonium fluoride, the phosphoramidite derivatives **74a**,**b** were synthesised.

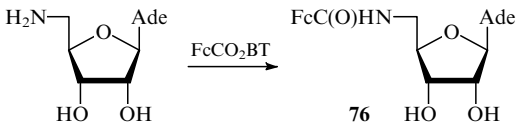


TBDPS = $Bu^tPr^i_2Si$; HOBT =  ; TBAF = $Bu_4\overset{+}{N}\ F^-$;
n = 2 (**a**), 4 (**b**).

The phosphoramidite derivative of 5′-ferrocenecarboxamido-5′-deoxythymidine **75** was synthesised in a similar way.



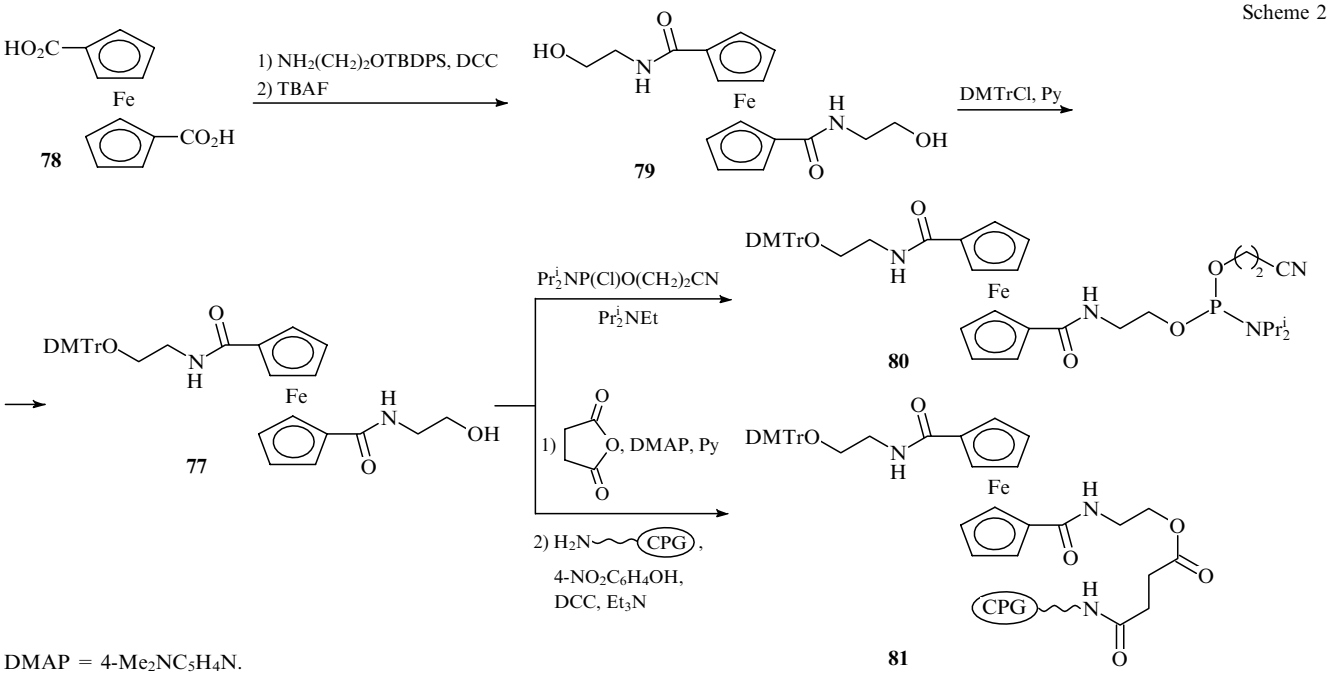
Synthesis of 5′-ferrocenecarboxamido-5′-deoxyadenosine **76** from *N*-hydroxybenzotriazole ester of ferrocenecarboxylic acid has been developed recently by Kraatz and co-workers.[78]



Synthesis of compound **77**, which can be used for incorporation of the ferrocene residue inside or at the end of the oligonucleotide chain, has been described.[79] Condensation of 1,1′-ferrocenedicarboxylic acid **78** with *O*-protected aminoethanol in the presence of dicyclohexylcarbodiimide and subsequent deprotection of the hydroxy groups afforded compound **79**; treatment of the latter with dimethoxytrityl chloride in pyridine affords the monoprotected derivative **77**. Compound **77** was converted into a standard building block for oligonucleotide synthesis (**80**) by phosphitylation. The reaction of the derivative **77** with succinic anhydride and subsequent immobilisation on a solid phase support containing aminoalkyl groups afford compound **81** (Scheme 2). It is known [80] that non-nucleoside inserts strongly destabilise DNA duplexes; therefore, compound **80** was attached to the 5′-end of the oligonucleotide in the last step of the synthesis (yield 50%). The ferrocene-containing fragment was attached to the 3′-end using compound **81**. The yield of the target oligonucleotide was 12% (possibly due to degradation of the ferrocene residue in the course of an automatic oligonucleotide synthesis, particularly during oxidation of phosphites into phosphates).

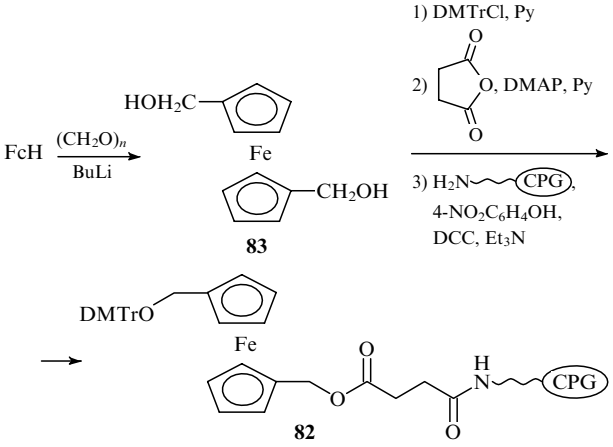
The same work [79] describes the use of a modified polymeric support **82** for the synthesis of oligonucleotide conjugates containing ferrocene at the 3′-end and of 5′-conjugates for inverted automatic oligonucleotide synthesis. Reaction of ferrocene with paraformaldehyde in the presence of butyllithium gave 1,1′-ferrocenedimethanol **83** in which one hydroxy group was protected by dimethoxytrityl chloride and the other one was acylated with succinic anhydride and immobilised on a solid phase support to obtain compound **82**. A phosphoramidite for incorporation inside the oligonucleotide chain can also be synthesised from the ferrocene derivative **83**.

Scheme 2



DMAP = 4-$Me_2NC_5H_4N$.

Synthesis of ferrocene conjugates with peptide nucleic acids has recently been described.[81, 82] It is known [83] that peptide nucleic acids form stable specific duplexes with DNA. These duplexes when they contain mismatches in base pairs are strongly destabilised.[81, 82] This property was used in the design of a DNA sensor.[61, 84]



n = 8–100.

III. Electrochemical detection of nucleic acids

The need for identification and quantitative determination of NA has stimulated the development of numerous analytical methods. Certain known procedures were adapted to the detection of these biomolecules. Electrochemical methods occupy an important place among other techniques for quantitative and qualitative detection of DNA.

Electrochemical activity of NA was discovered about 40 years ago;[85] the first evidence for the polymorphism of the double helix of DNA was confirmed some time later using electrochemical methods.[86]

Interaction of NA with a charged electrode surface is usually accompanied by strong absorption and concomitant charge transfer reactions. The electrochemical signals thus generated provide evidence about the type of adsorbed molecules, their concentrations, structural changes and an ability to interact with various compounds. Thus NA fragments immobilised on an electrode surface can be used in the design of NA detectors.[87] Native DNA is usually analysed using mercury or carbon (less frequently gold, platinum, copper or silver) electrodes. Solid metal electrodes are more suitable for the study of DNA oxidation; the oxidation of guanine and adenine is also carried out on carbon electrodes, whereas reduction is most conveniently studied on the surface of mercury electrodes. Heterocyclic bases are the main NA fragments involved in the redox reactions on electrodes.

Hydrogen bonds formed by complementary base pairs in electrochemically active DNA molecules influence strongly signals elicited during adenine and cytosine reduction on mercury electrodes and provide information about the presence or absence of complementary interactions on the electrode surface, structural transitions and local conformational changes in DNA molecules.[88, 89]

Redox reactions of native DNA are irreversible and require the application of high positive or negative potentials, which may cause unwanted destruction of DNA chains. The sensitivity of analytical methods can be increased through incorporation of electroactive reporter groups covalently or noncovalently linked to a single-stranded DNA or a DNA duplex. Such markers are capable of reversible redox conversions at low electric potentials on electrodes. Non-covalent binding of reporter molecules to natural DNA can involve: (1) electrostatic and, as a consequence, nonspecific binding to charged phosphate groups of DNA; (2) minor or major groove binding; and (3) intercalation of aromatic molecules between heterocyclic bases of a DNA duplex. Chelate complexes of transition metals and ferrocene derivatives are often used as markers.[90, 91]

There are two approaches different in principle to detect DNA. In one of them, complexes of stable isotopes of iron or other metals are used as labels. Incorporation of chemically identical labels carrying different isotopes of the same element into the DNA primers has virtually no effect on their physicochemical properties, *e.g.*, electrophoretic mobilities. This allows sequencing of extended NA fragments with high precision. Labelled DNA fragments are identified using resonance ionisation spectroscopy or mass spectrometry. The efficiency of this method for NA detection in the Sanger sequencing and amplification of DNA in the polymerase chain reaction (PCR) was demonstrated by Jacobson *et al.*[64, 65]. However, despite the significant progress in this field in the early 1990's, this method has not received wide recognition, because many reagents and materials used in molecular biological studies, *e.g.*, nylon membranes, contain iron which decreases drastically its sensitivity.[92]

An alternative approach consists of the use of amperometric techniques for detecting Fe(II)/Fe(III) redox signals. It should be noted that the use of ferrocene and its derivatives as markers permits directed changes in the halfwave potential ($E_{1/2}$) of the redox pair due to inductive effects of substituents in cyclopentadiene rings. This allows variations of electrochemical properties of reporter groups without any significant changes in their size or reactivity.[66]

1. Redox characteristics of ferrocene-containing nucleic acids

The value of the halfwave redox potential $E_{1/2}$ determined experimentally by amperometric methods is an important characteristic feature of molecules involved in redox processes.

Introduction of electron-donor groups into the cyclopentadiene ring of ferrocene decreases the $E_{1/2}$ value, whereas electron-withdrawing groups increase it as was demonstrated by Morris in 1974.[93] There are several groups of nucleoside and oligonucleotide ferrocenyl derivatives with similar redox potentials (Table 2). The simultaneous use of several ferrocenyl derivatives enables parallel detection of different NA fragments, *e.g.*, in a flow.

The change in the redox potential upon absorption of ferrocene-containing derivatives on the electrode surface is due to the increase in the dielectric component of the potential of the adsorbed molecule. A comparison of $E_{1/2}$ values for ferrocene oxidation in oligonucleotides (*e.g.*, at the end of an alkyl linker in the 2′-position of the carbohydrate fragment) and in a water-soluble ferrocenyl ethyl phosphate devoid of nucleotide units has been carried out.[57] It was found that electrochemical characteristics of ferrocene-containing oligonucleotides in solution differ only slightly from those of other ferrocene derivatives.

Studies by cyclic voltammetry have shown that the electrochemical characteristics of the ferrocene unit have virtually no dependence on either the structures of the complexes of ferrocene-containing oligonucleotides with DNA (a duplex or a triplex) or their nucleotide compositions.[68] A small (10 – 30 mV) decrease in the halfwave oxidation potential of the ferrocene unit at the 5′-end of a short oligonucleotide upon its incorporation into a triplex may be attributed to the interaction of the ferrocenyl cation with the phosphate groups as a result of which the former is less exposed to the solution. Thus, it was concluded [68] that ferrocene

Table 2. Redox potentials (*versus* Ag/AgCl) of conjugates of NA fragments with ferrocene.

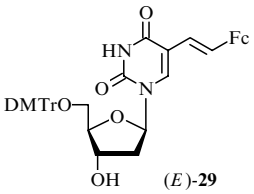
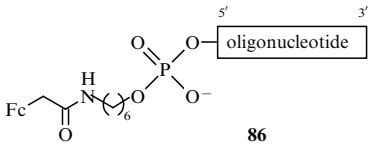
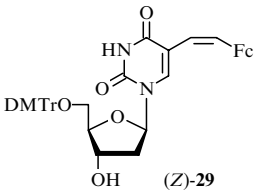
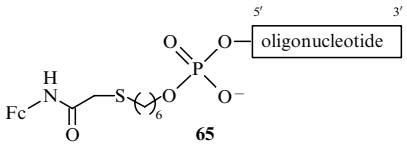
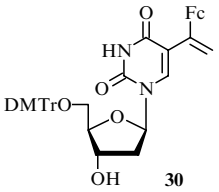
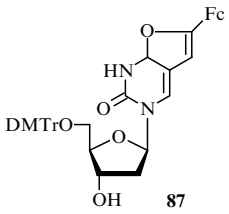
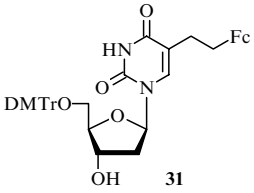
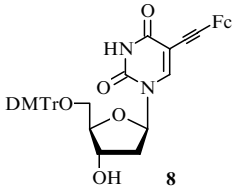
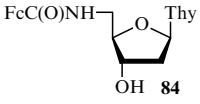
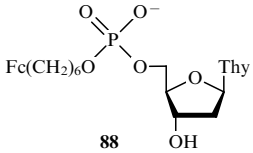
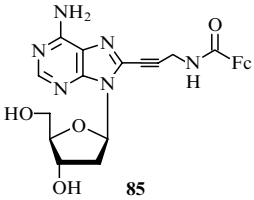
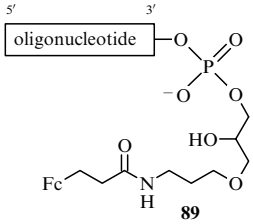
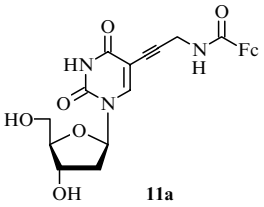
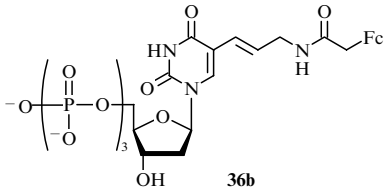
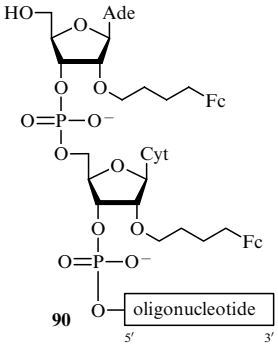
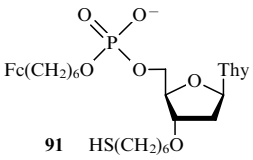
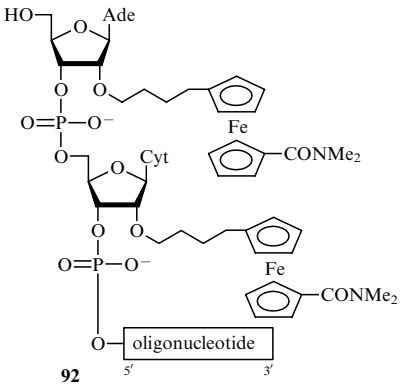
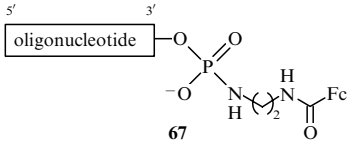
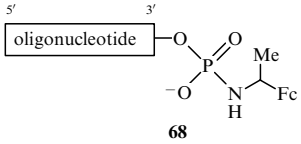
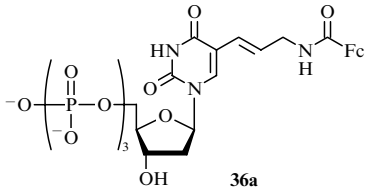
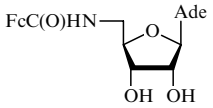
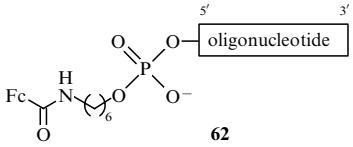
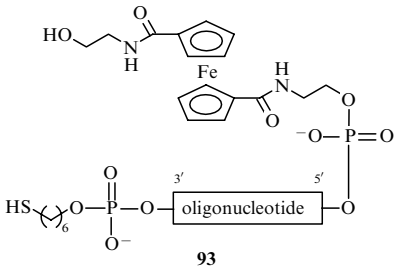
Compound	$E_{1/2}$ /mV	Ref.	Compound	$E_{1/2}$ /mV	Ref.
 (*E*)-**29**	27 [a]	39	 **86**	60	72
 (*Z*)-**29**	29 [a]	39	 **65**	16 [b]	73
 **30**	21 [a]	39	 **87**	131 [a]	39
 **31**	16 [a]	39	 **8**	140 [a]	39
 **84**	24	41	 **88**	110	76
 **85**	48	41	 **89**	135 [c]	94

Table 2 (continued).

Compound	$E_{1/2}$ /mV	Ref.
 **11a**	262	40
 **36b**	260	50
 **90**	181	57
 **91**	220 [d]	76
 **92**	324	58
 **67**	300	74
 **68**	290	75
 **36a**	398	50
 **76**	412	78
 **62**	430 645 [e]	68 70
 **93**	574 [d]	79

[a] Solution in acetonitrile. [b] *Versus* a standard carbon electrode. [c] *Versus* a saturated calomel electrode. [d] The nucleotide was immobilised on an electrode. [e] The oligonucleotide was hybridised with a target immobilised on an electrode.

can be used as a versatile electrochemical marker for detection and quantitation of DNA.

2. Electrochemical detection of ferrocene-containing DNA fragments and their complexes using HPLC analysis and capillary electrophoresis

High-performance liquid chromatography with UV detection is a popular conventional procedure for purification and analysis of NA [95, 96] owing to its high efficiency and a relatively low cost. Wlassoff and King [50] have carried out a comparison of various UV and electrochemical techniques for detecting ferrocene-containing oligonucleotides to demonstrate that the latter enable quantitative determination of DNA in femtomolar concentrations, while the sensitivity of the optical methods does not exceed picomolar concentrations (Table 3), *i.e.*, it is by about three orders of magnitude lower.

Takenaka *et al.*[67] have developed a highly sensitive method for detecting polynucleotides in solution by hybridisation with complementary oligonucleotides containing ferrocene residues at the 5′-end followed by HPLC analysis and electrochemical detection.

Table 3. Detection limits of ferrocene-containing oligonucleotides established by different analytical methods.

Method	Detection limit /mol (sample concentration /mol litre^{-1})	Ref.
HPLC with electrochemical detection	1×10^{-15} (1×10^{-9})	50
HPLC with electrochemical detection (hybridisation of a ferrocene-containing oligonucleotide with a target)	1×10^{-15}	67
HPLC with electrochemical detection, preliminary PCR	1×10^{-19}	69
Capillary gel electrophoresis with sinusoidal voltammetric detection	3×10^{-14} (3×10^{-9})	72
	1.8×10^{-15} (9×10^{-10})	97
Hybridisation of a ferrocene-containing oligonucleotide with a target bound to an oligonucleotide immobilised on an electrode; differential pulse voltammetry	1×10^{-15}	71
Hybridisation of a ferrocene-containing oligonucleotide with a target immobilised on a chitosan-coated glass electrode; differential pulse voltammetry	2×10^{-11} (2×10^{-9})	75
Hybridisation of a ferrocene-containing oligonucleotide with a target immobilised on a carbon electrode; differential pulse voltammetry	5×10^{-11} (5×10^{-9})	74

The sensitivity of this method is comparable with the detection limit of radioactive labels (10^{-15} mol, see Table 3). It is noteworthy that fluorescent and luminescent methods, which demand preliminary enzymatic amplification of DNA with modified nucleoside triphosphates, appeared to be more sensitive, but were more time-consuming in comparison with electrochemical methods (2–3 days *vs.* 2–3 hours). HPLC with electrochemical detection was successfully used [67] for quantitation of a series of natural polynucleotides (transport gene of yeast choline, oncogen *v-myc*, eukaryotic mRNA). The analysis of mRNA is facilitated by the presence of a 3′-terminal poly-A sequence, which enables the use of homothymidine sequence as a signal oligonucleotide.

An improved procedure for the detection of DNA in solution developed in 1997 [69] is based on the combination of HPLC [67] and PCR, which makes it possible to increase the number of DNA copies using ferrocene-containing oligonucleotides as primers.[98] The amplification products were analysed by HPLC with electrochemical detection, which significantly lowered the detection limits (see Table 3). Yet another advantage of this method is that the analysis does not require the use of control DNA samples, whereas in routine quantitative analyses using PCR, DNA amplification is performed in the same tube with DNA under analysis. This method was used for quantitation of the oncogen *v-myc* and the human muscular dystrophin gene.

Capillary gel electrophoresis has been extensively used over the past decade for the analysis of DNA. It represents a standard and the most efficient procedure for DNA sequencing.[99] This method combined with the use of fluorescently labelled NA has received wide acceptance. Detection by mass spectrometry is also becoming very popular.[100] In recently published papers,[8, 101] capillary electrophoresis of PCR products (ferrocenyl derivatives of NA) was carried out with electrochemical detection.[72, 97]

The authors of a device for electrochemical detection of biomolecules in capillary gel electrophoresis by an amperometric method suggested that electrochemical labels were directly incorporated into the molecules to be analysed.[8] Such an approach allows simultaneous detection of different molecules and is analogous to fluorescence detection at several wavelengths.

Capillary gel electrophoresis with sinusoidal voltammetric detection [102] was used for selective identification of DNA amplification products.[97] This method is characterised by high signal/noise ratios and maximum selectivity in detecting electrochemically active molecules based on their unique frequency spectra. Oligonucleotides **62**, **86**, **90** and **92** containing ferrocene residues in their 5′-terminal units were used as PCR primers. The electrochemical originality of each of the four oligonucleotide primers was achieved owing to the presence of substituents in the ferrocenyl fragments able to influence the redox potential of ferrocene. This, in turn, enables detection and analysis of distribution patterns of ferrocene-containing oligonucleotides in electrophoretic fractions. In addition, cyclic voltammetric studies have shown that static voltammograms of ferrocene-containing primers virtually do not differ from those recorded under electric field in the electrophoresis. This allows one to extend the scope of the method, since it does not require the use of additional corrections for an electric field.

It was recommended [72] to use sinusoidal voltammetry for the analysis of single base extension products of an oligonucleotide chain by capillary gel electrophoresis. This was carried out by hybridisation of a DNA probe with a ferrocene-containing primer followed by addition of 2′,3′-dideoxynucleoside triphosphate (ddNTP) complementary to the nucleotide under study and thermosequenase, a thermostable Ampli Taq polymerase from *Thermus aquaticus* (Fig. 1). The extension reaction stopped in the case of a point mutation in the DNA sample. The unreacted primer was separated from the reaction product by capillary gel electrophoresis.¶ The fractions were analysed by sinusoidal voltammetry. This method allows easy miniaturisation, which makes it a convenient tool for the development of an automatic portable system for detecting mononucleotide polymorphism in the genome.

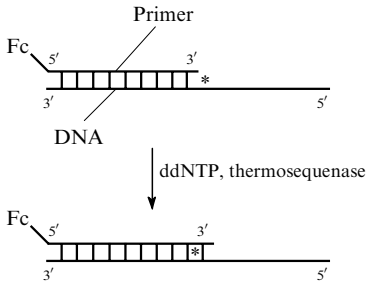


Figure 1. A scheme of a one-unit elongation reaction of an oligonucleotide chain.[72]

3. Electrochemical detection of ferrocene-containing DNA fragments and their complexes on the electrode surface

A biosensor (or a biodetector) consists of an element that selectively recognises biologically active molecules and is connected to a sensor which converts molecular recognition into a physically measurable quantity.[103] A standard electrochemical detector for DNA hybridisation represents an electrode with short (as a rule, 15–20-mer) oligonucleotides immobilised on its surface (Fig. 2). When such an electrode is placed into a solution containing DNA to be sequenced, a duplex is formed on the electrode surface. The changes in the surface layer are detected by electrochemical or gravimetric methods. The choice of the elec-

¶ It should be noted that this method allows separation of oligonucleotides differing in one unit and containing up to 250 bases.

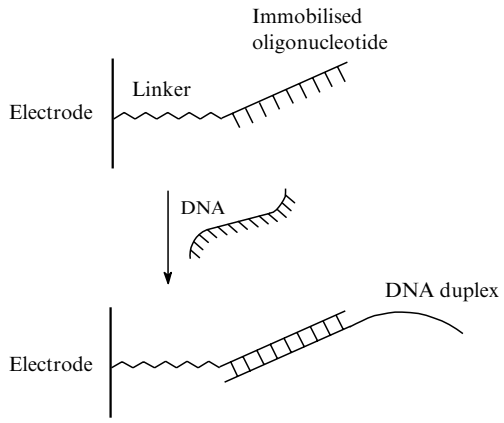


Figure 2. The formation of a duplex on the electrode surface.

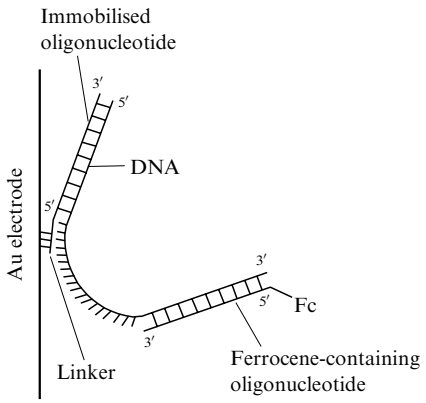


Figure 3. The formation of a three-component sandwich-like system immobilised on a gold electrode.[70]

trode material and the immobilisation procedure are crucial steps in the design of DNA sensors.

Immobilisation of oligonucleotides is performed by various methods which include covalent and noncovalent binding to the electrode surface. Thus immobilisation on carbon and mercury electrodes is carried out by the electrostatic adsorption method. Adsorption on positively charged electrodes is achieved owing to electrostatic attraction of negatively charged phosphate groups, which leaves heterocyclic bases accessible for the interaction with specific DNA sequences.

Covalent immobilisation of oligonucleotides on the electrode surface, which involves reactive groups of heterocyclic bases, is hardly recommendable because of a decrease in sensitivity of DNA sensors. The attachment of one end of an oligonucleotide molecule to the electrode surface is the most efficient approach. In the general case, covalent immobilisation of oligonucleotides is more convenient for the design of biosensors, since it permits one to avoid nonspecific binding of electrochemically active molecules.[87]

At present, chemisorption of oligonucleotides on an electrode surface makes use of both modified oligonucleotides and electrodes with a modified surface.[104]

In 1996, Letsinger *et al.*[76] developed a procedure for electrochemical characterisation of a monolayer formed by molecules of an immobilised nucleotide **99** on the surface of a gold electrode. This approach entails incorporation, into the nucleotide, of both a 3′-terminal linker with a thiol group essential for immobilisation and a 5′-terminal ferrocene residue. The monolayer formed after chemisorption of such molecules on the electrode surface was studied by cyclic voltammetry. The data obtained suggest that the surface density of nucleotide molecules on gold is 3.0×10^{-10} mol cm^{-2}. The applicability of such systems for detection of DNA hybridisation has been demonstrated.[76]

An original gene diagnostics procedure has been developed.[70] In this method, several phosphothioate groups were introduced into the 5′-terminal region of an oligonucleotide complementary to a specific site of the DNA target and the oligonucleotide was immobilised on a gold electrode. After hybridisation with DNA, the electrode was treated with a solution of a ferrocene-containing oligonucleotide **62** complementary to the free region of the DNA molecule (Fig. 3). The detection was performed using differential pulse voltammetry. If a solution contained a DNA target complementary to both fragments, a sandwich-like system was formed on the surface of a gold electrode, which resulted in the appearance of a peak of anodic oxidation of the ferrocene unit of the oligonucleotide **62**. The concentration of the DNA fragment to be assayed was ~ 50 μmol litre^{-1}. In control experiments with a nucleotide sequence different from that of the DNA target and an oligonucleotide containing only one mismatch within the recognising seven-nucleotide site, the anodic oxidation peak of ferrocene was virtually absent.

Later, immobilisation of the phosphothioate-containing oligonucleotide followed by hybridisation with a DNA probe and the signal oligomer **62** were studied in more detail[71] using quartz crystal microgravimetry. The density of the molecules on the electrode surface calculated from the results obtained was equal to 77 pmol cm^{-2}. Taking into account the magnitude of the square per ferrocene molecule within the complex with DNA ($\sim 200-700$ Å^2), a conclusion was drawn that the density of the molecules inside the layer was rather high. Since the roughness of the electrode surface was neglected in this study, the theoretically calculated density of the layer package could be significantly overestimated. This, in turn, can be the reason for excessive charge density and a significant (by 215 mV) increase in the redox potential of the ferrocene-containing oligonucleotide **62** in comparison with its oxidation potential in solution (see Table 2). An increase in its potential can be attributed to the destabilisation of the oxidised form of ferrocene in the conjugate, since the DNA chain prevents the approach of counterions to the ferrocenyl cation because of increased density of anionic phosphate groups. This finding is consistent with the results of Letsinger *et al.*[76] which suggest that the difference in the redox potential values of ferrocene in the immobilised oligonucleotide and in solution amounts to 110 mV. This approach appeared to be extremely sensitive to one-letter substitutions.[71]

This method was modified further (Fig. 4).[58, 59, 61, 62, 105] A gold electrode, following immobilisation of an oligonucleotide, was treated with 16-mercaptohexadecanol in order to protect the

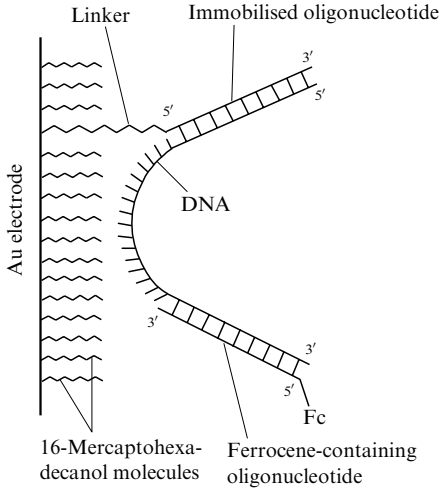
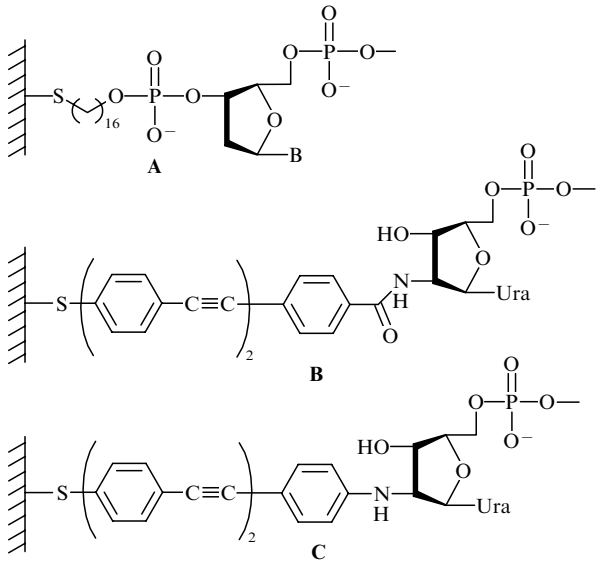


Figure 4. A modified version of an electrochemical analysis of DNA.[58, 59]

electrode surface from the molecules that can nonspecifically be absorbed and thus violate the results of measurements. This improves the efficiency of measurement.

Immobilisation of oligonucleotides on the surface of a gold electrode was performed using three types of linkers,[58, 59, 61, 62] *viz*., a standard linker A and linkers B and C which improve the electron transfer:[106]



Immobilisation of sulfur-containing oligonucleotides on the surface of a gold electrode was used in the design of a DNA chip for detecting mutant fragments of DNA in solution at a concentration of ~ 50 nmol litre^{-1}.[58] Two ferrocene-containing oligonucleotides differing in their oxidation potentials and complementary to DNA regions of natural and mutant types were used (the probe was taken in a 5–10-fold excess with respect to the DNA under study). Hybridisation of these oligonucleotides with DNA was carried out at an elevated temperature which was optimum for the formation of perfect duplexes. Ferrocene-containing DNA duplexes were detected by alternating current voltammetry. It was found [58] that such a system enables reliable identification of mononucleotide polymorphism.

The same group of investigators [105, 107] have developed a new procedure for electrochemical detection of DNA using DNA chips.[58] This method is based on a comparison of temperature dependences of signal intensities on voltammograms for the formation of perfect and imperfect duplexes with a ferrocene-containing oligonucleotide. With an increase in temperature, imperfect duplexes become unstable, which ensures reliable identification of mononucleotide polymorphism of extended (up to 100 bases) DNA fragments. The advantage of this method includes long-term operation of biosensors without any loss in electrochemical signal intensity.[105, 107] Such DNA chips were recommended for detection of mononucleotide substitutions in human DNA samples.[108, 109] The use of DNA chips based on ferrocene-containing oligonucleotides in clinical diagnostic procedures has been surveyed in detail.[110, 111]

An alternative approach to immobilisation of oligonucleotides has been recently proposed by Houlton and co-workers [112, 113] who performed direct synthesis of NA on a surface further used as a support.

It is known [114, 115] that unfolding of hairpin structures of NA can be favoured by interactions with completely complementary oligonucleotides. Detection and analysis of hairpin structures are usually performed with the help of a fluorophore and a fluorescence quencher attached to the opposite ends of the oligonucleotide hairpin (Fig. 5). The formation of a duplex, which competitively interacts with the hairpin, increases the distance between the fluorophore and the fluorescence quencher which results in the enhancement of fluorescence. Such a scheme was

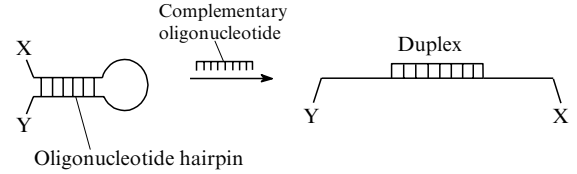


Figure 5. Unfolding of an NA hairpin structure in the presence of a complementary oligonucleotide.[114, 115]
X is fluorophore; Y is fluorescence quencher.

implemented in the case of an oligonucleotide immobilised on gold nanoparticles [116] used as fluorescence quenchers.

The same approach was used in the design of a DNA sensor (Fig. 6).[45, 79] The hairpin immobilised on a gold electrode retains its structure in the presence of mismatching oligonucleotide fragments, while a duplex is formed in the presence of a matching DNA sequence. As a result, the distance between the ferrocene and the electrode increases which reflects on the efficiency of the electron transfer and, as a consequence, on the current.

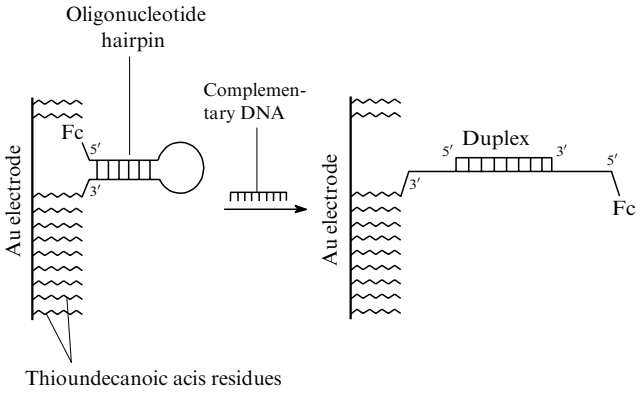


Figure 6. Electrochemical detection of DNA using a hairpin oligonucleotide.[45, 79]

A method for detecting DNA on the electrode surface [74] consists of the following. Single-stranded extended DNA fragments that are analysed are immobilised on a carbon electrode by applying an electric potential of $+0.5$ V as described by Wang *et al*.[117] This is followed by hybridisation with electrochemically active DNA fragments containing a residue of ferrocenecarboxylic acid. The resulting conjugates are analysed by differential pulse voltammetry.

Later, this method was optimised.[75] For the first time, the carbon electrode was replaced by a glass electrode coated with chitosan, a polysaccharide containing amino groups able to electrostatically interact with negatively charged phosphate residues of DNA. This ensures the strongest binding of DNA under study to the electrode surface. A complementary DNA containing a 3′-terminal ferrocenyl residue is used as a probe. After hybridisation, the signal of ferrocene oxidation was recorded by differential pulse voltammetry. In this case, the detection limit could be lowered to 2×10^{-11} mol (see Table 3) and the selectivity remained high. However, the ability of DNA immobilised on chitosan due to electrostatic interactions to undergo hybridisation with the probe to form a double helix without disturbing these interactions is questionable.

An interesting application of ferrocene-containing oligonucleotides immobilised on a gold electrode as a model in studies of DNA flexibility has been described.[94]. A 20-mer homothymidine oligonucleotide containing a thiol group at the 5′-end and a ferrocene unit at the 3′-end was used as a model DNA. This

oligomer manifests minimum rigidity in comparison with other sequences. After hybridisation of the immobilised oligonucleotide with a complementary template, the mobility of the 3′-terminal fragment containing a ferrocene residue decreased as could be evidenced from cyclic voltammetry data. At high sweep rates, the peak of ferrocene oxidation disappeared from the voltammograms.

A procedure for detecting ferrocene-containing molecules by a flow injection method has been developed.[118] Here, quantitative analysis of electroactive molecules is performed in a buffer solution containing potassium hexacyanoferrate(II), the so-called 'sacrificial reagent' which yields hexacyanoferrate(III) upon oxidation. Direct oxidation of the 'sacrificial reagent' on the surface of a gold electrode is excluded due to preliminary immobilisation of an alkanethiol monolayer (Fig. 7). The hydrophobic ferrocene moieties attached to the analyte can be incorporated into the alkanethiol monolayer. If a potential is applied, the ferrocene immobilised on the electrode surface begins to act as a catalytic mediator of hexacyanoferrate(II) oxidation which is recorded by voltammetry. This method has sufficiently low detection limits for ferrocene detection (an amplification factor of about 300 in comparison with direct measurement of ferrocene-containing molecules in solution).

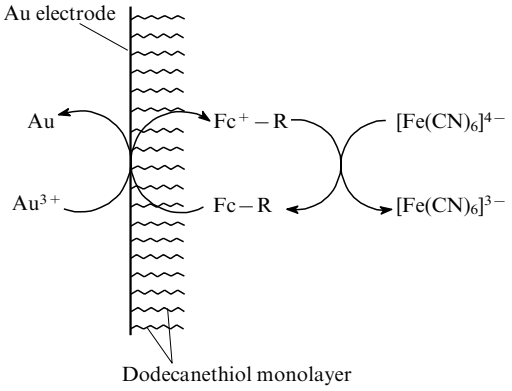


Figure 7. Detection of ferrocene-containing molecules by a flow injection method.[118]
R is a molecule to be quantified.

In the general case, HPLC and capillary electrophoresis coupled with electrochemical detection (see Table 3) allow attainment of lower limits for DNA detection in solutions than immobilisation of oligonucleotides on electrodes. Nevertheless, the latter approach seems to hold much promise for the detection of one-letter substitutions.

IV. Conclusion

A variety of methods for attachment of the ferrocene residue at the 5′- and 3′-ends and inside the oligonucleotide chain are presently available. Some of them enable incorporation of ferrocenyl derivatives during automatic oligonucleotide synthesis, while others are used for postsynthetic modification. The choice of an optimum procedure is determined by the experimental task; preference is given to postsynthetic attachment of ferrocene. In the first place, this is due to the commercial availability of nucleosides and oligonucleotides containing reactive groups, since ferrocene-containing nucleosides and their phosphoramidite derivatives are commercially unavailable. Moreover, under standard conditions of an oligonucleotide synthesis ferrocene undergoes partial destruction in the oxidation stage of phosphite groups and during postsynthetic workup in alkaline media. The problem of ferrocene degradation in the oxidation step can be solved by replacing iodine by milder commercially available oxidants, such as *tert*-butyl hydroperoxide or (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine. It is also recommended to use phosphoramidite nucleoside derivatives with labile acetyl or phenoxyacetyl protective groups which can be removed under very mild conditions. This allows one to increase the yields of modified ferrocene-containing oligonucleotides.

Preparation of modified nucleoside triphosphates is yet another trend in the synthesis of ferrocene-containing NA fragments. It has been shown [119] that nucleoside triphosphates with substituents at C(5) of pyrimidine or at C(7) of purine bases are ideal modified substrates for DNA polymerase and RNA polymerase. Earlier, it was found [120] that 2′-deoxyuridine triphosphate derivatives modified at C(5) with propynyl or (*E*)-propenyl groups are substrates for Taq polymerase unlike (*Z*)-propenyl and propyl derivatives. Based on these data, two synthetic strategies can be distinguished: (1) synthesis of a ferrocene conjugate with a modified nucleoside triphosphate residue containing a chemically reactive group and (2) alkynylation or alkenylation of a nucleoside by a ferrocene-containing derivative and its subsequent conversion into a triphosphate. However, account must be taken of the fact that many nucleoside triphosphate (*E*)-propenyl [50] and propynyl [121] derivatives are not substrates for DNA polymerases and individual testing of each triphosphate of the modified nucleoside may be required.

The possibility of incorporating a ferrocene residue into a predetermined position of an oligonucleotide chain opens up broad opportunities for the development of diverse systems for electrochemical detection of nucleic acid fragments. There are two approaches to the analysis of DNA. One of them consists of enzymatic synthesis of NA by PCR and makes use of triphosphates of ferrocene-containing nucleosides or 5′-ferrocene-containing primers. The product is further analysed by HPLC or capillary electrophoresis with electrochemical detection. This may also include hybridisation of a ferrocene-containing oligonucleotide with an NA target and detection of the duplex formed. This approach enables detection (including quantitation) of very small concentrations (down to attomoles) of polynucleotides.

The second approach includes detection of DNA on the surface of electrodes containing immobilised oligonucleotides bearing covalently linked ferrocene residues. The use of various detection schemes holds great promise for the analysis of SNP in extended DNA fragments. To obvious advantages of this method one can relate its high sensitivity and selectivity; however, in this case larger amounts of NA are required.

Electrochemical detection is far less efficient than the fluorescent method where parallel capillary electrophoresis is used. However, it is a promising tool in the design of DNA chips. DNA chips, eSensor™ (Motorola Clinical Micro Sensors, Pasadena, CA, USA) and an eSensor™ 4800 Reader allowing DNA analysis simultaneously on 48 chips, have been developed based on original publications.[57–59, 105]

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