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Affinity sorbents containing nucleic acids and their fragments

I G Shishkina, A S Levina, V F Zarytova

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Abstract. The published data on the main methods for the preparation of polymeric supports containing nucleic acids (NA) or their fragments (oligonucleotides) are reviewed with special emphasis on chemical immobilisation. Some physical and physicochemical immobilisation techniques, including those based on the use of enzymes and avidin – biotin interactions and preparation of NA-containing supports by direct oligonucleotide synthesis on these supports are considered. A special section is devoted to the application of NA-containing sorbents for the isolation of individual NA and proteins as well as in hybridisation analysis including those utilising DNA chips and DNA biosensors. The bibliography includes 391 references.

I. Introduction

Polymeric supports containing nucleic acids (NA) and their fragments have long attracted the attention of specialists in different fields of biochemistry, gene engineering and molecular biology. In the past decade, such supports have successfully been used in hybridisation analysis for research purposes as well as in medicine for the diagnostics of infectious, cancer, genetic and other diseases. NA-Containing matrices are efficient tools for isolation of individual NA and NA-dependent enzymes. The synthesis of affinity sorbents is based on the following principle: the ligands (in our case, NA, oligonucleotides or polynucleotides) are immobilised on a polymeric support (a matrix) through a linker or a spacer, or without them. With the advent of automated synthetic techniques, oligonucleotides have become commercially readily available, which makes them especially attractive as ligands. Native NA are also immobilised as ligands for the solution of some practical tasks.

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All presently known methods for immobilisation of oligonucleotides and NA can be conventionally divided into chemical methods, which involve covalent binding of NA to the support, and other methods, which include all the procedures demanding special equipment or technology (*e.g.*, synthesisers, irradiation, *etc.*) in addition to the support, NA and chemicals.

This review is devoted to the description of the main methods for the preparation of NA-containing sorbents, their properties and applications. It should be noted that these methods can be used for immobilisation of not only oligo(poly)nucleotides, but also their analogues and peptide-nucleic acids (PNA) in which the sugar-phosphate backbone is replaced by the peptide chain. A separate section is devoted to the use of NA-containing matrices for the isolation of individual NA and proteins as well as in hybridisation analysis (including DNA chips and DNA biosensors).

II. Solid supports and linkers

1. Supports

Before we pass to the consideration of various procedures for immobilisation of NA, we shall dwell briefly on the requirements placed upon polymeric supports to be used in the design of 'ideal' affinity matrices. These include:

(1) insolubility (to avoid the loss of the ligand attached and contamination of the isolated compound with the dissolved support);

(2) accessibility of the immobilised ligand for the formation of complexes with specific biomolecules;

(3) zero adsorption capacity (to prevent non-specific interactions);

(4) high reactivity essential for chemical modification of the support surface;

(5) chemical stability under conditions employed for the attachment of ligands as well as sorption and desorption of the products and regeneration of the matrix;

(6) high resistance to microbes and enzymes;

(7) hydrophilicity (to reduce non-specific sorption due to hydrophobic interactions);

(8) commercial availability.

The requirements for the nature, shape, composition and mechanical strength of the solid support vary depending on the experimental task. The most popular supports can be divided into two groups, *viz.*, natural and synthetic ones (Table 1). High-

Table 1. Commercially available carriers.

Carrier	Commercial name	Manufacturer
Natural supports		
Agarose	Sepharose, Superose	Pharmacia, LKB
	Bio-Gel A	Bio-Rad
	Ultrogel	IBF
Cellulose	Cellulose	Whatman
Dextran	Sephadex	Pharmacia
Synthetic supports		
Polyacrylamide supports	Bio-GelP	Bio-Rad
	Trisacryl GF-2000, Sephacryl	IBF Biotechnics
	Ultrogel	Pharmacia
	Azlactone beads	IBF
	3M Emphaze biosupport	3M Corporation
		Pierce Chemical
Polymethacrylate supports	TSK-Gel Toyopearl HW	Tosoh Corporation
	Fractogel TSK	EM Science, Merck
	HEMA	TESSEK Ltd.
	Separon, Spheron	Alltech Associates
	Eupergit	Rohm Pharma
Polystyrene supports	Porous supports	PerSeptive Biosystem
	Polystyrene balls	Pierce Chemical
	Dyno particles	Trondheim
	TentaGel	Rapp Polymers
	MerckoGel	Merck
Glass supports		
	CPG glass	Pierce Chemical
	Glass beads	Jenco, Supelco
	Microscope glass slides	Fisher Scientific
		Menzel-Glaser

pressure affinity chromatography demands hard materials, whereas in column chromatography performed at normal pressure hardness is not a critical factor. The relatively small specific surface area in non-porous supports can largely be compensated by the reduced size (down to the micron level) of the support particles with the preservation of their satisfactory hydrodynamic characteristics. Porous supports are suited to affinity chromatography where the size of the analysed molecules is much smaller than that of the matrix pores. In the majority of cases, sorbents represent spherical granules with the size of 40 to 150 μm, whereas silica gel-based sorbents for high-performance affinity chromatography have a particle size of about 10 μm.

Modern studies often utilise membranes as supports. They are usually manufactured from various polymeric materials, such as cellulose, polyamide (nylon), polyacrylonitrile, polysulfone, polypropylene, polyesters, polyethylene, *etc*. Cellulose, nylon and poly(ethylene terephthalate) are the most popular supports, since they allow easy incorporation of various functional groups. Cover glasses and polystyrene immunological assay plates are widely used in hybridisation analysis.

Some supports incorporating various functional groups (Table 2) accessible for immobilisation of ligands are commercially available; however, they demand additional modification prior to application because they partly lose their activities upon storage. Therefore, we shall consider some synthetic procedures leading to such sorbents along with the principal known methods for the preparation of affinity sorbents containing NA or their fragments.

2. Linkers and spacers

Oligonucleotides and NA can be immobilised directly on the surface of the solid support; however, this can result in steric hindrance to subsequent reactions with biomolecules, especially in the case of planar supports, such as membranes and slides. To prevent this, immobilisation is carried out with the use of linkers or spacers which hold the ligand at some distance away from the surface and thus make it more accessible for further interactions.

In the absence of linkers, immobilisation can be carried out by virtually all physical methods (*e.g.*, UV irradiation, sorption on the surface by drying) or by direct synthesis of oligonucleotides on glass [1] or on aminated polypropylene.[2] However, immobilisation is usually performed after incorporation of spacers into the oligonucleotide and/or on the surface of the support.[3–40] The larger is the distance between the immobilised ligands and the surface, the more accessible they are for the reaction with the dissolved molecules. Most often, the role of linkers is played by linear aliphatic chains containing different numbers of hexamethylenediamine, 6-aminocaproic acid or ethylene glycol units. In addition to the length, an important role is played by the nature of the linker, which may govern the extent of non-specific sorption. This can be overcome through the use of linker chains consisting of hydrophilic molecules, *e.g.*, 1,3-diaminopropan-2-ol or amino acid residues. Hydrophilic linkers of any length can be designed from phosphoroamidites of various diols [8] or by stepwise addition of 1,3-diaminopropan-2-ol and bromoacetic acid.[9,10] The linkers are usually attached to the support by the same methods as ligands. Silica gel and glass are premodified by (3-aminopropyl)-triethoxysilane (**1**),[11–13] (3-glycidyloxypropyl)trimethoxysilane (**2**),[3,4,13–15] [*N*-(2-aminoethyl)-3-aminopropyl]trimethoxysilane (**3**), (diethylenetriaminopropyl)trimethoxysilane (**4**)[16] or other trialkoxysilanes.[17–21] The number of immobilisation sites can be increased by an order of magnitude using branched linkers, such as dendrimers (**5**).[22]

Oligonucleotides with linkers (3–11 units) attached to their 5′- (see Refs 7, 23–28) or 3′-terminal [15,29] phosphates, to the apurinisation sites,[30] to the amino group of 5-methyldeoxycytidine,[31,32] to C(5) of deoxyuridine [33–36] or to C(8) of deoxyadeno-

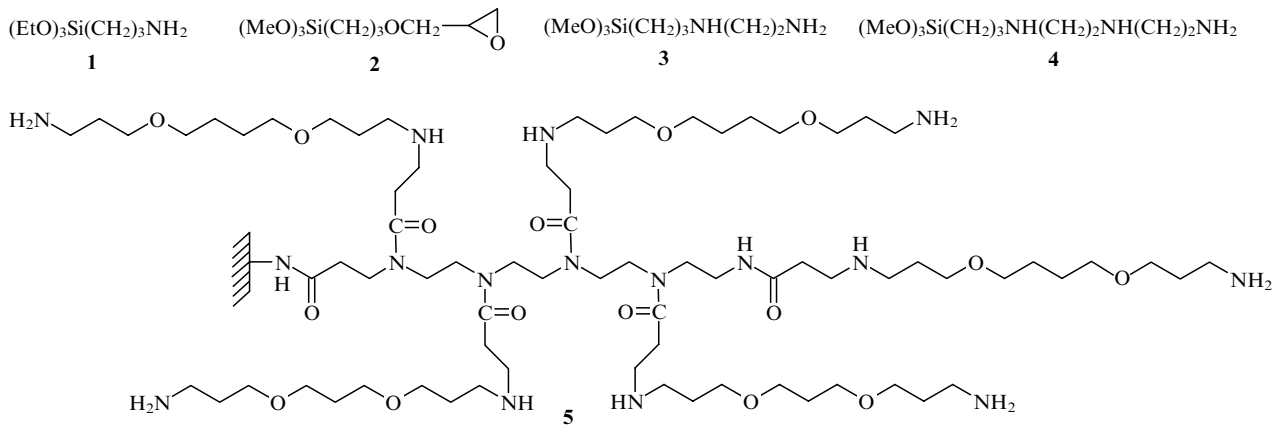
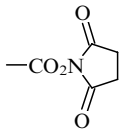
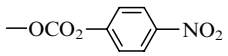
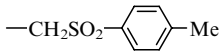
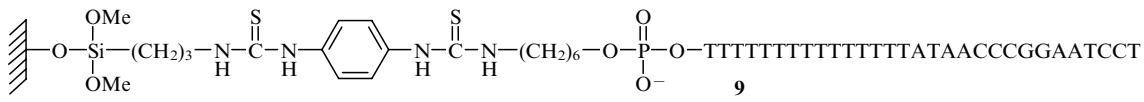


Table 2. Supports for the immobilisation of ligands.

Functional groups of supports	Ligand	Groups of supports formed after immobilisation of the ligand	Commercial name of the support	Manufacturer
—$CONHNH_2$	LCHO	—C(O)NHN=CHL	Affi-Gel Hz gel, Affi-Prep Hz support	Bio-Rad
			Agarose adipic acid hydrazide	Pharmacia
			Carboxymethylcellulose hydrazide	Sigma, Supelco
			3M Emphaze hydrazide biosupport	Pierce
			Hydrazide beads	Pierce, Sigma, Supelco
			Polyacrylamide hydrazide	Sigma, Supelco
			Polyacrylhydrazidoagarose	Pierce
—$OC(O)NH(CH_2)_2NHCO$—C$_6$H$_4$—HgCl	LSH	—$OC(O)NH(CH_2)_2NHCO$—C$_6$H$_4$—HgSL	Immobilized *p*-chloromercuribenzoate gel	Pierce
			Affi-Gel 501gel	Bio-Rad
—$OCH_2CH(OH)CH_2SS$—C$_5$H$_4$N	LSH	—$OCH_2CH(OH)CH_2SSL$	Thiopropyl-activated	
			Agarose	Sigma, Supelco
			Sepharose 6B	Pharmacia, Sigma, Supelco
			Thiolagarose	ICN
—O(O)C—O—C=NCHCONHCHSS—C$_5$H$_4$N (CO$_2$H; C(O)NHCH$_2$CO$_2$H)	LSH	—O(O)C—O—C=NCHCONHCHSSL (CO$_2$H; C(O)NHCH$_2$CO$_2$H)	Activated thiolsepharose 4B	Pharmacia, Supelco
—$NH(CH_2)_3NHC(NH)(CH_2)_3SS$—C$_6H_3$(CO$_2$H)(NO$_2$)	LSH	—$NH(CH_2)_3NHC(NH)(CH_2)_3SSL$	Immobilized TNB-thiol	Pierce
—$CH_2NHCOCH_2I$(Br)	LSH	—$CH_2NHCOCH_2SL$	Ultralink iodoacetyl	Pierce
			Bromoacetylagarose	ICN, Calbiochem
			Bromoacetylcellulose	Sigma
—O(O)C—O—C=NH	LNH_2	—O(O)C—O—C=NL	Cyanogen bromide-activated	
			Agarose, Sepharose 6M B	Sigma, Supelco
			Agarose CL-4B	Fluka
			Sepharose 4B	Fluka, Sigma, Supelco, Pharmacia
—$OCH_2CH(O)CH_2$	LOH LNH_2	—$OCH_2CH(OH)CH_2XL$ X = O, NH	Epoxy-activated	
			Agarose	Pierce, Sigma, Supelco
			Sepharose 6B	Pharmacia, Sigma, Supelco
			Eupergit C	Rohm Pharma, Supelco
			Oxirane acrylic beads	Sigma
			Polymercarrier VA-epoxy	Riedel-de Haen
			POROS 20 EP	PerSeptive Biosystem
			Supelcosil epoxy 540	Supelco
			Toyopearl AF-epoxy-650M	Supelco, Tosoh Co.

Table 2 (continued).

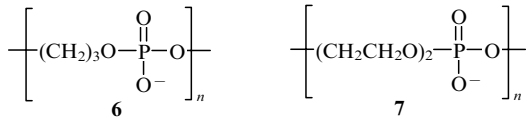
Functional groups of supports	Ligand	Groups of supports formed after immobilisation of the ligand	Commercial name of the support	Manufacturer
	LNH_2	—OC(O)NHL	*N*-Hydroxysuccinimide-activated	
			Agarose	Sigma, Supelco
			CH-Sepharose	Fluka, Sigma, Supelco
			Sepharose, Superose	Pharmacia
			Affi-Gel, Affi-Prep support	Bio-Rad
	LNH_2	—OC(O)NHL	Nitrophenyl-activated Agarose	Sigma, Supelco
	LNH_2	—OC(O)NHL	Carbonyldiimidazole(CDI)-activated	
			Agarose	Sigma, Supelco
			Trisacryl GF-200, TSK HW-65F	IBF Biotechnics
			Reacti-Gel (6X, GF-2000, HW-65F) Supports	Pierce
	LSH LNH_2	—CH_2XL X = NH, S	Tosyl-activated	
			Agarose	Pierce, Sigma, Supelco
			Dynabeads M280	Dynal Inc.
—$CH_2OSO_2CH_2CF_3$	LSH LNH_2	—CH_2XL X = NH, S	Tresyl-activated Agarose	Pierce, Schleicher & Schuell, Sigma, Supelco
			Toyopearl AF-tresyl-650M	Supelco, Tosoh Co.
—OCH_2CHO	LNH_2	—OCH_2CH_2NHL	Toyopearl AF-formyl-650M	Tosoh Co.
			Aldehydeagarose	Sigma



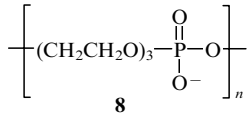
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sine [37] are immobilised on commercially available supports (see Table 2) bearing relatively short linkers (2–11 units).

There is no general agreement as regards the dependence of hybridisation properties of immobilised oligonucleotides on the linker's length. It was noted [6, 16] that the hybridisation abilities of immobilised oligonucleotides are enhanced with increase in the linker's length. It is also suggested [8, 38, 39] that efficient hybridisation requires the optimum length of the spacer to be selected. The use of linkers (**6**–**8**) containing different numbers of repeating units has also been documented.[8]



6 **7**



8

The maximum efficiency of hybridisation is achieved for $n = 8-10$ with all three types of units. This suggests that the yields of hybridisation products depend not only on the length of the linker, but also on its structure (in this case, probably, on the number of negative charges). The maximum increase in the yields of hybridisation products (150-fold in comparison with the yields in the absence of the spacer) was observed with the linkers **7** and **8** for $n = 8$. With a further increase in the number of units, the hybridisation ability diminishes, and for $n = 25-30$ the yields of the hybridisation products are virtually the same as in the absence of the linker.

However, it was shown [40] that the length of the linker between the polyacrylamide support and an oligonucleotide does not influence the stability of a duplex formed. This is consistent with the results of the above-cited studies and those devoted to the sequencing of DNA by hybridisation on oligonucleotide microchips.[41–47] Presumably, the optimum length of the linker depends on many factors, such as the nature of the support, the ligand and the linker itself, and should be chosen experimentally for each particular task. It is noteworthy that immobilisation on granulated supports can be made using short linkers or no linkers at all, whereas films and slides demand longer linkers.

In order to increase the distance between the surface and an oligonucleotide to be immobilised, the latter sometimes is elongated by introducing additional chains consisting of thymidine units [*e.g.*, oligo$(dT)_{1-400}$][17, 19, 48, 49] or non-essential oligonucleotide sequences (*cf.* **9**).[7, 18]

III. Chemical methods for the immobilisation of nucleic acids

Immobilisation of NA by covalent binding to polymeric supports is achieved by the reaction of available or incorporated functional groups of the oligonucleotide with reactive groups of the support. In this review, the chemical methods used for the formation of a covalent bond between the ligand and the polymer are divided into two large groups, *viz.*,

(1) immobilisation of functionalised and/or activated NA on nucleophilic supports, and

(2) immobilisation of NA on supports containing electrophilic groups.

It should be noted that the functional groups of the natural nucleic acids (hydroxy groups of ribose, phosphoric acid residues and heterocyclic bases [50]) possess nucleophilic properties and react with the electrophilic groups of the polymer. These reactions usually yield numerous binding sites of the ligands to the supports, which results in the loss of the full-value interactions of immobilised NA with biomolecules. To prevent this, NA are modified by introduction of additional functional groups which are more reactive than the corresponding functional groups of the natural NA.

1. Immobilisation of functionalised and activated nucleic acids on nucleophilic supports

Immobilisation of NA and their fragments on solid supports containing surface nucleophilic groups (*e.g.*, hydroxy, thiol or amino groups) can be achieved by preliminary activation of the functional groups of the ligand (Fig. 1).

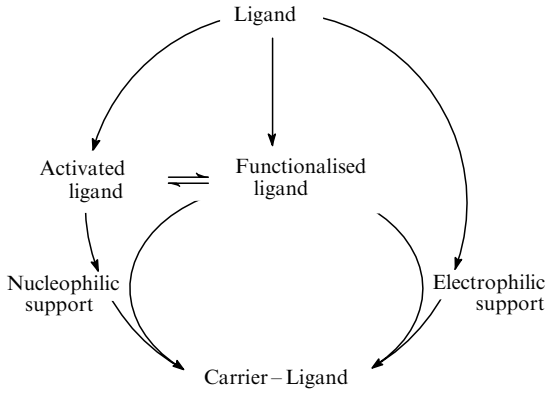


Figure 1. Chemical methods for the immobilisation of ligands.

The introduction of functional groups into poly- and oligonucleotides has been the subject of several reviews.[51–53] Most often, the post-synthetic introduction of additional functional groups involves terminal phosphate groups using reagents which affect other functional groups of NA to a much lesser extent. Carbodiimides, arenesulfonyl chlorides, 2,4,6-trimethylbenzoyl chloride, *etc.*, are commonly used for this purpose.[54–58] The reactive oligonucleotide derivatives formed are unstable and used for the introduction of additional functional groups into oligonucleotides or for direct immobilisation on solid supports immediately following preparation.

a. Activation of nucleic acids by carbodiimides

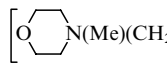
The addition of NA using carbodiimides **10a**–**c** has been one of the earliest approaches to the preparation of affinity sorbents and has continued to be a very popular procedure.[50–64] This method is based on the ability of carbodiimides to activate either the carboxy groups of polymers or the terminal phosphate group of NA. Carbodiimide-activated oligonucleotides react readily with amino or thiol groups of solid supports. Reactions with hydroxyl-containing supports proceed less effectively.

The carbodiimide method is used for the immobilisation of mono-, oligo-, polydeoxyribonucleotides and DNA in both organic solvents using dicyclohexylcarbodiimide (**10a**)[50–62] and aqueous media using water-soluble carbodiimides, *e.g.*, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (**10b**)[13, 18, 24, 25, 65, 66] or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (**10c**).[25, 67]

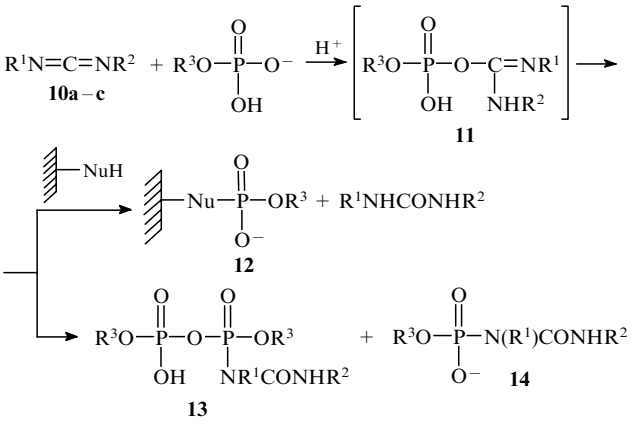
$R^1N{=}C{=}NR^2$

10a–c

$R^1 = R^2$ = cyclo-C_6H_{11} (**a**); R^1 = Et, $R^2 = Me_2N(CH_2)_3$ (**b**);

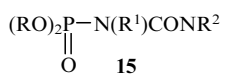
R^1 = cyclo-C_6H_{11}, R^2 =  $[SO_3C_6H_4Me\text{-}4]^-$ (**c**).

The mechanism of activation of phosphate groups of oligonucleotides by carbodiimides has been studied in detail and some side reactions have been revealed.[68–73] Protonation of carbodiimides and the formation of intermediate unstable reactive *O*-phosphorylisourea derivatives **11** is the limiting step of this reaction.[74] This stage is also inhibited by strongly basic nucleophilic groups of the support. Besides, protonated nucleophilic groups do not react with compounds **11**. As a consequence, the yield of the final product **12** decreases if polymers with strongly basic groups are used. The substituted *O*-phosphorylisoureas **11** can also be transformed into side products, *e.g.*, be hydrolysed with the liberation of the original phosphate group and urea or undergo consecutive conversions leading to pyrophosphates, polyphosphates and *O*-phosphorylisourea derivatives.[75] The latter, in turn, produce non-reactive *N*-pyrophosphorylurea derivatives **13**[75,76] or *N*-phosphorylurea derivatives **14**.[66,77–79]



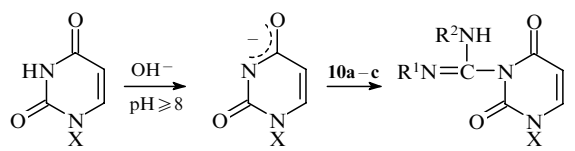
R^3, oligonucleotide residue; NuH = NH, SH, OH.

In the activation of oligonucleotides by carbodiimides resulting in compounds **11**–**14**, modification of internucleotide phosphate groups[76,79] and heterocyclic bases[69,80] is observed. In the case of oligodeoxyribonucleotides, the reaction with the internucleotide phosphate groups results in the urea derivatives **15**,[76] whereas the reaction with oligoribonucleotides leads to the isomerisation and cleavage of the internucleotide bonds.[51,81]



15

The activation of the terminal phosphate group of the nucleotide by carbodiimide in alkaline media (pH $\geqslant$ 8) can be accompanied by nucleophilic addition of the anion formed from the heterocyclic base to the double bond of the carbodiimide. This reaction affects the bases containing a CONH group, *i.e.*, the N(3) atom in the uracil and thymine residues and the N(1) atom in the guanine residues.[69]



X, oligonucleotide residue.

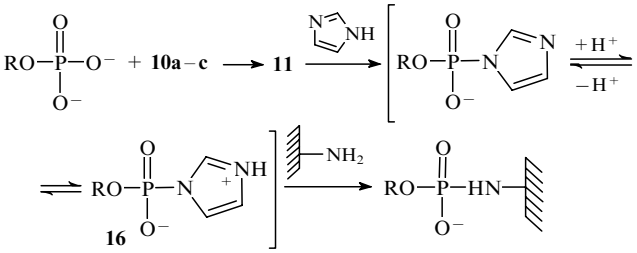
The rate of the limiting stage can be accelerated, the yield of the target product **12** in the immobilisation of the oligonucleotide derivatives **11** can be increased and the modification of the internucleotide phosphodiester groups and heterocyclic bases by carbodiimides can be overcome if the reaction is carried out in weakly acidic media (pH 4.25–5.0).[80] In this case, the carbodiimide-activated terminal phosphate group of an oligonucleotide reacts predominantly with the amino, thiol or hydroxy groups on the surface of the support.

The selectivity and efficiency of oligonucleotide immobilisation increases slightly after functionalisation of their 5′-phosphate groups by linkers containing a terminal carboxy group,[18,82] which reacts with the amino groups of the support after its activation by carbodiimide.

An alternative approach consists in the activation of the carboxy groups of the polymer by carbodiimides (see Section III.2). In this case, immobilisation of nucleotides and oligonucleotides on supports involves the amino groups of the heterocyclic bases or linkers attached to the 3′- (see Refs 66, 83) or 5′- (see Refs 25, 84) terminal phosphates of the oligonucleotides.

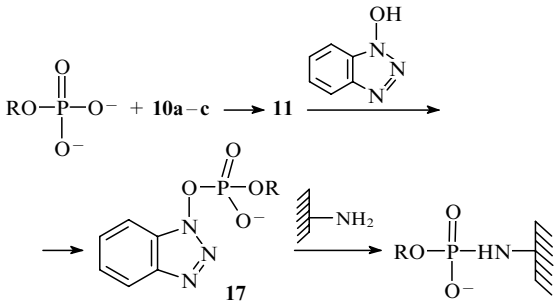
Immobilisation of NA and oligonucleotides by the carbodiimide method is carried out on virtually all available solid supports, *e.g.*, cellulose, Sepharose,[59–63,72,85] Sephacryl,[23,65,67] Sephadex,[23,73,85] SiO_2-based supports[12,18,64,65,86,87] and various membranes.[66,82,83,88] In addition to conventional supports, magnetic carriers (*e.g.*, polystyrene-coated magnetite particles)[25] and latex microspheres (polystyrene beads able to form colloidal suspensions)[24] are used. The resulting matrices are used in hybridisation analysis[12,13,18,23,25,66,67] and in affinity chromatography.[63,89]

The efficiency of immobilisation of oligonucleotides on polymeric supports can be increased by performing activation by carbodiimides in the presence of nucleophilic catalysts, *e.g.*, imidazole, triazole or tetrazole, which generate the corresponding phosphoramidates;[24,25,65,73,88] the latter manifest high reactivities towards nucleophiles after protonation of the azole units. Phosphorimidazolidates **16** are commonly used.[88,90,91]



R, oligonucleotide residue.

The addition of compounds containing hydroxy groups with higher acidities, *e.g.*, *N*-hydroxysuccinimide or *N*-hydroxybenzotriazole, to the reaction mixture instead of nucleophilic catalysts results in the formation of the activated NA esters **17**.[73,92]

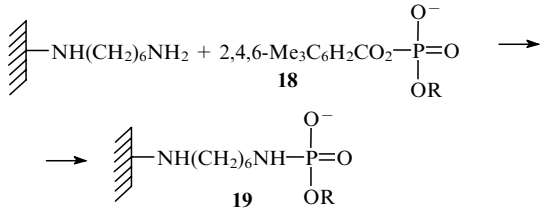


R, oligonucleotide residue.

The main advantage of the last two approaches is the lack of side reactions, since the derivatives **16** and **17** are more stable than the *O*-phosphorylisoureas **11**. The compounds **16** and **17** react with the amino groups of the support more efficiently, which increases the yield on immobilisation.

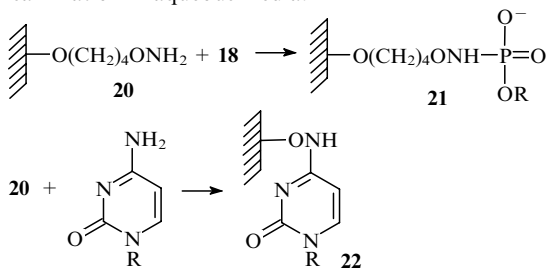
b. Activation of nucleic acids by 2,4,6-trimethylbenzoyl chloride

High reactivity of the title reagent with respect to the terminal and internucleotide phosphate groups ensures rapid formation of the corresponding mixed anhydrides. In aqueous media, only the mixed anhydride **18** formed upon reaction with the terminal phosphate persists.[93,94] This is used for the immobilisation of deoxyribonucleic acids on polymers containing nucleophilic amino groups. In aqueous media, immobilisation is due to the formation of a phosphoramide bond between the terminal 5′-phosphate group of the oligonucleotide and the primary amino group of the polymer (the product **19**).[57,95]



R, oligonucleotide residue.

In the study by Shumyantseva and Khomutov,[96] the activated oligonucleotide **18** was immobilised on aminooxybutylcellulose **20**. The resulting affinity sorbent **21** is stable only at pH 7.0 due to the high lability of the alkoxyamide bond. In addition, binding of oligonucleotides to the polymer **20** occurred involving the amino groups of cytidine to give the product **22**, most probably due to transamination in aqueous media.[56,94]

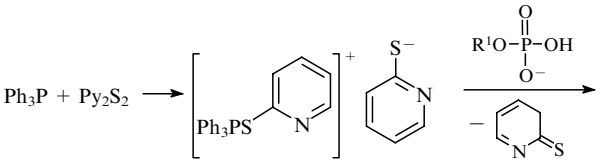


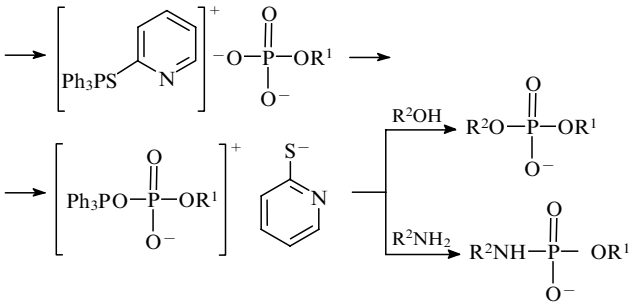
R, oligonucleotide residue.

This method has not gained wide acceptance despite the fact that TMP, AMP, CMP, d(pT)$_3$, d(pA)$_3$ and other oligonucleotides[57,95,96] were immobilised on polysaccharide supports in fairly good yields (0.05–0.15 mmol of the nucleotide per 1 g of the support). The significant disadvantages of this method are the lengthy immobilisation step (> 3 days) and side reactions (*e.g.*, the formation of pyrophosphates of oligonucleotides, reactions involving internucleotide phosphates and the heterocyclic bases of oligonucleotides). This method is inappropriate for the immobilisation of ribooligonucleotides and RNA, since mixed anhydrides favour the formation of cyclophosphates in the activation step, subsequent isomerisation and cleavage of the internucleotide bonds.[93]

c. Activation of the terminal phosphates of oligonucleotides by the triphenylphosphine–dipyridyl disulfide redox couple

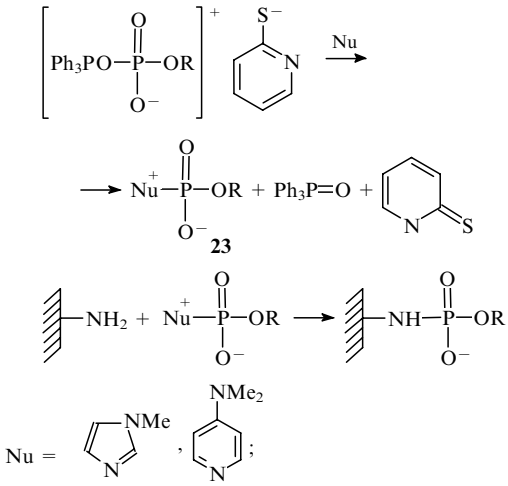
The triphenylphosphine–dipyridyl disulfide redox couple ($Ph_3P-Py_2S_2$) was first recommended for use as a condensation reagent for peptide and oligonucleotide syntheses by Mukaiyama *et al.*[97,98] The following reaction scheme was proposed:[98]





R^1, nucleotide or oligonucleotide residue; R^2 = Alk, Ar.

However, the reaction of an oligonucleotide with highly basic amino groups in the presence of the Ph_3P-PyS_2 couple occurs at a slow rate and with low yields, which places certain restrictions on its practical application. A detailed study of this system by Zarytova *et al.*[99–102] revealed that the $Ph_3P-Py_2S_2$ couple selectively activates the terminal phosphate group of an oligonucleotide in the presence of nucleophilic catalysts with the formation of the zwitterionic oligonucleotide derivatives **23**.



R, nucleotide or oligonucleotide residue.

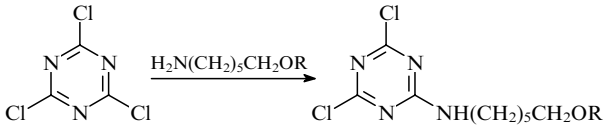
It should be emphasised that heterocyclic bases and internucleotide phosphate groups are not involved in the activation, which precludes the formation of side products. Therefore, this approach can be used for the activation of both deoxyoligonucleotides and their analogues and, which is especially important, of ribooligonucleotides without migration of the C(3)–C(5′) internucleotide bond.

The activated derivatives **23** react rapidly and virtually quantitatively with primary amines to yield oligonucleotide phosphoramides.[100] The activated oligonucleotide **23** can easily be immobilised provided the primary amino group is localised on its surface.[101,102] The immobilisation is completed within 2 h in aqueous alkaline media or in organic solvents. Up to 97% of the starting oligonucleotide material binds to the support, which enables one to control and vary the capacities of affinity sorbents. Any material can be used as a support, but silica gel[103–105] and polymethacrylate gels[101,106] are especially widely used. The matrices with immobilised oligonucleotides were used for affinity chromatography of NA-dependent proteins,[101,106] nucleic acids and their analogues.[102–105] The $Ph_3P-Py_2S_2$ couple can be recommended for the immobilisation of ribooligonucleotides and RNA, since in contrast with the known condensation reagents (*e.g.*, carbodiimides, arenesulfonyl chlorides, azoles, 2,4,6-trimethylbenzoyl chloride), it does not react with internucleotide phosphates and nucleophilic centres of heterocycles and no corresponding side products are formed.

d. Activation of nucleic acids by bifunctional reagents

Activation and subsequent immobilisation of NA and their fragments can be achieved through the use of bifunctional reagents. The reactions with bisoxiranes, disuccinimidyl carbonate, carbonyldiimidazole and glutaraldehyde will be considered in detail in Section III.2.

The bifunctional reagents also include 2,4,6-trichloro-1,3,5-triazine (**24**), which reacts with the primary amino group of a spacer introduced into the oligonucleotide in the course of its synthesis.[27]



24 **25**

R, oligonucleotide residue

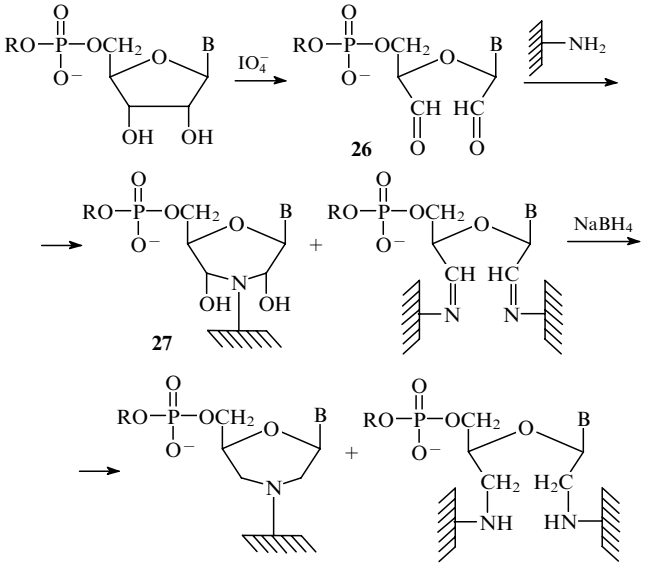
The triazine derivatives of oligonucleotides **25** are stable for at least one week at 4 °C and can be immobilised on any supports containing amino (see Refs 6, 27) or thiol groups.[6] The resulting sorbents are characterised by low non-specific sorption of proteins and NA and are stable under hybridisation conditions.[27, 107]

e. Reductive amination with nucleic acids and oligonucleotides containing aldehyde groups

Reductive amination is based on the well-known reaction of aldehydes and ketones with primary amines resulting in the formation of Schiff's bases. The reduction of Schiff's bases by sodium borohydride affords stable secondary amines.

$$R^1NH_2 + O{=}CHR^2 \rightleftharpoons R^1N{=}CHR^2 \xrightarrow{NaBH_4} R^1NHCH_2R^2$$

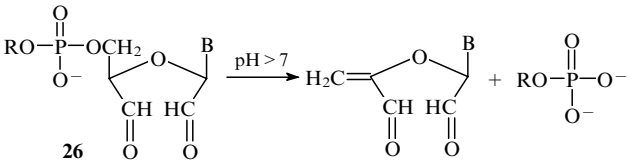
Immobilisation based on this method requires that NA or oligonucleotides contained aldehyde groups. This requirement is easy to meet in the case of ribooligonucleotides, RNA and deoxyoligonucleotides carrying a 3′-terminal ribose moiety. The *cis*-diol group of the terminal ribose is selectively oxidised by sodium periodate. The aldehyde groups formed (compound **26**) react with supports containing surface amino [108–111] and hydrazide [112–114] groups.



R, ribooligonucleotide, RNA or deoxyoligonucleotide;
B, heterocyclic base.

In this reaction, the dialdehyde can react both with one amino group of the support (to give the morpholine derivative **27** [50, 52]) and with two amino groups leading to bisadducts. Since the derivatives of the type **27** are stable only at pH > 9 and phosphate undergoes β-elimination at pH < 8, they should be reduced with sodium borohydride or cyanoborohydride immediately after preparation.

A decrease in the yield of the immobilisation products can also be due to the cleavage of the 5′-C—O bond in the periodate oxidation product **26** in alkaline media (β-elimination) resulting in the splitting of the modified nucleoside from the 3′-end of RNA.[50]



26

Immobilisation on supports containing hydrazide groups occurs with higher yields, since hydrazones do not require treatment with reductants, being more stable than azomethines.[29] It should be noted, however, that the binding of an oligonucleotide to the support through the aliphatic amino group formed upon reduction is stronger [29] than that formed through the hydrazone group.

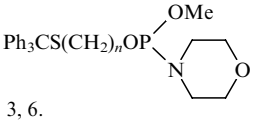
Reductive amination was used for efficient immobilisation of all ribonucleoside mono-, di- and triphosphates [114–118] on cellulose and agarose (Sepharose) modified by amino or hydrazide groups. The sorbents formed were used in affinity chromatography of proteins, such as myosins [118] and the elongation factors Tu and Ts.[110] Reductive amination is the main technique in immobilisation of ribonucleotides. Polyribonucleotides [poly(A) and poly(U)] following oxidation of the *cis*-glycol groups were attached to polysaccharide supports.[111, 115, 119] Among numerous studies aimed at immobilisation of RNA by this method,[112, 116, 119–121] special mention should be made of those studies [115, 117, 122, 123] in which ribosomal RNA were covalently attached to hydrazide-agarose and used for the isolation and purification of ribosomal proteins. tRNAs immobilised on polysaccharide templates [111, 115, 124] were used for the purification of aminoacyl-tRNA synthases.[125–128]

Recent developments in oligonucleotide synthesis have stimulated studies aimed at the synthesis of oligodeoxyribonucleotides containing a terminal ribonucleoside unit. Its 3′-*cis*-glycol group is oxidised and used for immobilisation of oligodeoxynucleotides on solid supports.[29, 64, 129, 130] The affinity matrices thus prepared are widely used in hybridisation analysis and sequencing of DNA.[42, 43, 45, 131–133]

Immobilisation of NA and their fragments by reductive amination can also be achieved by the introduction of the aldehyde groups into the supports. One can synthesise polymers of virtually any type containing reactive aldehyde groups. The methods of their synthesis will be discussed in Section III.2.i.

f. Immobilisation of nucleic acids containing thiol groups

The introduction of chemically reactive thiol groups into NA and their fragments [53] allows specific immobilisation of such ligands by conventional methods, *e.g.*, on supports containing epoxy groups (Section III.2.b) or active halogen atoms (Section III.2.e).[134] Special procedures can also be used for this purpose. Thiol groups can be introduced in the last step of the phosphoramidite oligonucleotide synthesis, *e.g.*, using commercial phosphorylating reagents, such as phosphoramidites of *S*-protected thio alcohols.[49, 134, 135]

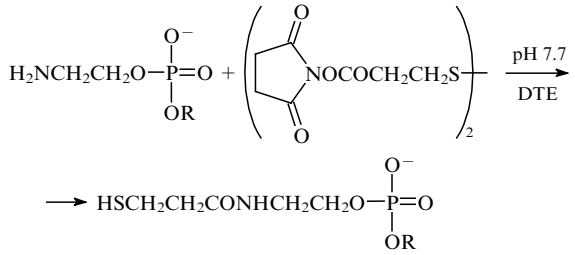


n = 3, 6.

The removal of all protective groups (including the trityl group) by silver nitrate leads to an oligonucleotide containing a

5′-terminal thiol group.[49, 135] Thiol groups can either be introduced into the 3′-end of an oligonucleotide in the initial stage of the synthesis,[16, 136] or incorporated into the isolated oligonucleotide by an enzymic reaction, *viz*., using ATP containing a γ-thiol group.[137]

An alternative approach consists in preliminary incorporation of an easily modifiable aliphatic amino group into the terminal 5′-phosphate group of an oligonucleotide.[90] The reaction of the spacer amino group of the deblocked oligonucleotide with 3,3′-dithiobis-*N*-(propionyloxy)succinimide in the presence of a disulfide bond reductant, *e.g*., dithioerythritol (DTE), dithiothreitol (DTT), cysteine or mercaptoethanol, affords an oligonucleotide containing a thiol group.



R, oligonucleotide residue; DTE, dithioerythritol.

Oligonucleotides containing thiol groups (SH-oligonucleotides) can be selectively immobilised on solid supports containing thiol groups, *i.e*., through the formation of a disulfide bond. Prior to immobilisation, the thiol groups of oligonucleotides can be activated by 2,2′-dipyridyl disulfide.[6]

If the supports and oligonucleotides contain non-activated thiol groups, the formation of the disulfide bond between NA and the matrix occurs under the action of oxidants (I_2 or H_2O_2).[49] Polysaccharides, glass (CPG),[90] magnetite particles coated with a silicon-containing polymer (Biomag),[49] polyacrylamide (Trisacryl, Sephacryl)[28] and polymethacrylate[6] are widely used as supports. Owing to the ease of the formation and the cleavage of the disulfide bonds by oxidising and reducing agents, thiol matrices are often used for reversible immobilisation of oligonucleotides containing thiol groups.

Immobilisation of purified SH-oligonucleotides proceeds rapidly and specifically. The resulting matrices manifest high stability and purity in hybridisation analysis[28, 49, 90] and in the purification of DNA-dependent proteins.[137] It should be noted, however, that the susceptibility of the thiol groups to oxidation creates storage problems. In the course of time, intermolecular disulfide bonds and side products can be formed. Immobilisation of SH-oligonucleotides on activated or functionalised supports will be considered below (Section III.2.f).

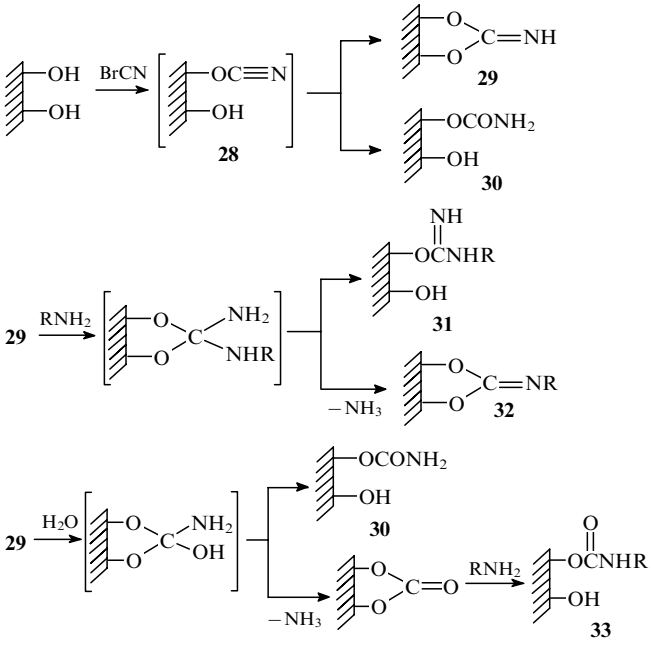
2. Immobilisation of nucleic acids on electrophilic supports

Native non-modified NA and their fragments are immobilised on functionalised and activated supports. Such supports can also be used for immobilisation of NA containing primary aliphatic amino or thiol groups introduced either in the course of or following the synthesis. The methods for the incorporation of the functional groups on the surface of supports able to react with the corresponding groups of the ligand will be discussed below.

a. Activation of supports by cyanogen bromide

A method of the activation of supports containing hydroxy groups by cyanogen halides was first proposed in 1967 for immobilisation of proteins and peptides.[138] The activation is usually carried out by cyanogen bromide at pH 10–11.[139–141] The reactive cyanate **28** formed upon activation reacts with the free hydroxy group of the support to give the reactive cyclic imidocarbonate **29** and the inactive carbamate **30**.[142, 143] A significant contribution to the development of this method was made by Cuatrecasas[144] who has selected the conditions for the immobilisation of various ligands including NA.[145]

In the early 1970's, this method became a routine procedure for the immobilisation of NA.[84, 139, 140, 146–150] The attachment of NA occurs at the amino groups of heterocyclic bases of a polynucleotide. In this way, polyuridylic[147, 149] and polyadenylic[139] acids and RNA[84, 141, 150] have been immobilised. After immobilisation of NA, the remaining reactive groups of the support are blocked by treatment with 1 M 2-aminoethanol.



It was shown that the isourea derivatives **31** were the main products formed in the immobilisation of a single-stranded DNA (0.2 mol litre^{-1} of 2-morpholinoethanesulfonic acid, pH 8.0) in formamide[139, 145]; the *N*-substituted imidocarbonates **32** and carbamates **33** are formed in lesser amounts.

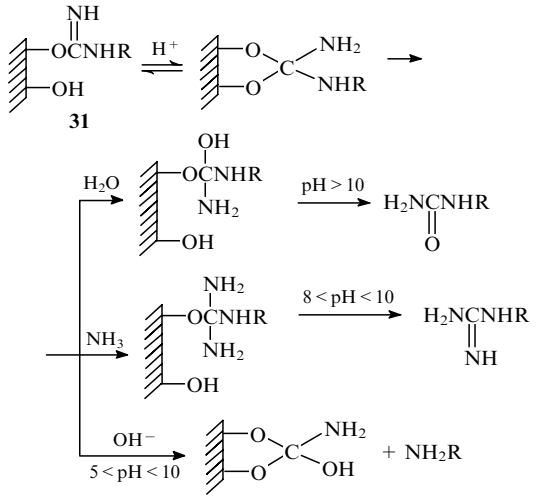
This method is most commonly used for the immobilisation of single-stranded DNA[139, 140, 146, 151, 152] and RNA.[84, 139, 141, 150] It was possible to prepare polymeric supports of high capacity and specificity despite possible reaction of the amino groups of heterocycles with activated supports. However, the affinity properties of the sorbents containing short oligonucleotides are weak, since the NH_2 groups of the heterocyclic bases involved in the covalent binding to the polymer cannot take part in the complex formation.

Immobilisation of poly- and oligonucleotides on cyanogen bromide-activated supports is much more selective when the linkers containing aliphatic primary amino groups are attached to either the 5′- or the 3′-end of the oligonucleotide chain[54–58, 153] or the internucleotide phosphate group.[154] However, in this case, too, partial immobilisation of the ligand occurs through heterocyclic bases.[155]

Nucleoside mono-, di- and triphosphates, such as AMP,[156, 157] ADP,[158, 159] ATP,[160] UMP,[58, 161] GTP[58] and dCp_4 (see Ref. 162), were immobilised on cyanogen bromide-activated Sepharose (all the nucleotides contained linkers with primary aliphatic amino groups incorporated into the phosphate groups or the heterocyclic bases). These sorbents were used for the purification of ribonucleotide reductase of phage T4,[160] the cap-binding protein[163] and deoxynucleotide kinase of *Lactobacillus*.[162]

The disadvantages of this method are as follows. First, immobilisation of the ligand must be carried out immediately after the activation of the support because of the low stability of the reactive groups formed upon treatment with cyanogen bromide. Second, cyanogen bromide is a toxic substance. Third, the basic isourea groups of the support **31** (pK_a = 9.5) formed by the reaction with the primary amine appear to be positively charged at physiological pH values, which confers undesirable anion-exchanging properties on such affinity sorbents. Moreover, this reaction yields numerous side products, while the binding sites of

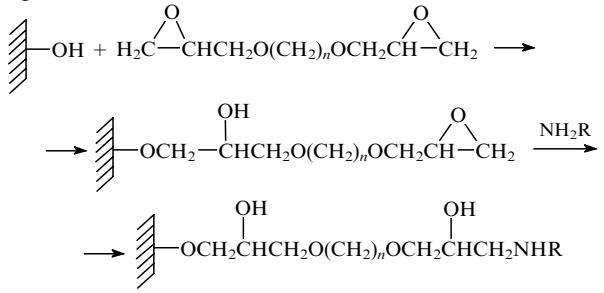
nucleotides cannot be determined with certainty. On storage, the ligand is gradually split off from the polymeric support.[113, 146, 164] It was shown [153] that affinity matrices prepared by immobilisation of oligothymidylates containing 5′-terminal amino groups on cyanogen bromide-activated cellulose retain their stabilities in aqueous media during one week at 22 °C and for only 24 h at 42 °C. Most probably, this is due to the instability of the *N*-substituted isourea derivatives **31**.[143]



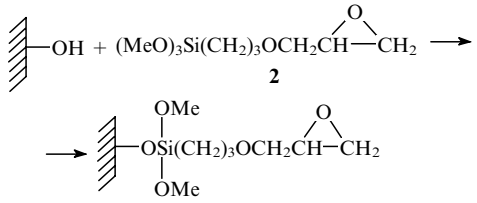
However, despite these drawbacks this method is widely employed for the immobilisation of DNA, RNA and synthetic oligonucleotides [165–170] aiming at the preparation of affinity matrices which are further used for the isolation and purification of DNA-dependent proteins.

b. Activation of supports by oxiranes

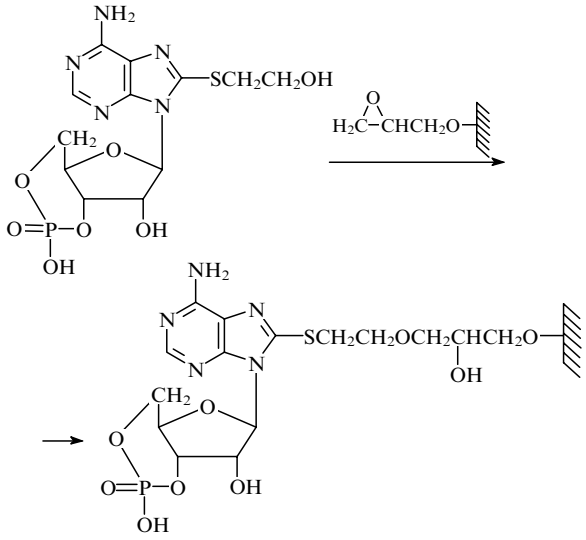
Bifunctional oxiranes were first recommended for the incorporation of reactive groups into polymeric supports and the stabilisation of gels by cross-linking.[171] The hydroxy groups of supports are usually activated by the diglycidyl ether of 1,4-butanediol at pH > 7.0.[37, 172, 173] This treatment affords a polymer containing reactive epoxy groups, which react with the nucleophilic groups of the ligands.



Epichlorohydrin is also used for the preparation of supports containing epoxy groups.[78, 107, 114] At present, epoxy groups are often incorporated on silica gel [3, 174] and glass [15, 175] using trimethoxysilanes **2** rather than polysaccharides.



As a rule, immobilisation of ligands containing amino, hydroxy or other nucleophilic groups on the supports containing epoxy groups occurs smoothly at pH 9–13 (8–20 h, 20–25 °C). The unconsumed epoxy groups are blocked by treatment with 2-aminoethanolamine (1 mol litre^{-1}). This method was used for the covalent binding of polynucleotides and some DNAs.[37, 172, 173, 176] The efficiency of binding of polynucleotides changed in the series: poly(dT) > poly(dC) = poly(dA) > poly(dG).[172] As nucleotides usually contain many functional groups, the exact binding site for polynucleotides is difficult to establish and therefore this is not always specified.[172, 173] For example, it is believed [37] that the immobilisation of the C(8)-hydroxyethylthio derivative of cyclo-AMP on a polymeric support containing epoxy groups is achieved by its binding through the terminal hydroxy group of the linker.

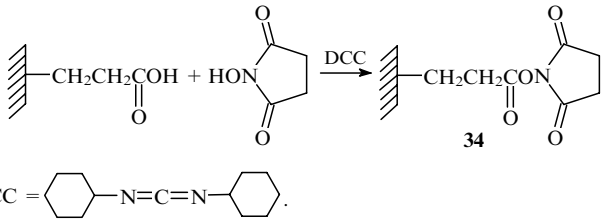


It was shown that the immobilisation of oligodeoxyribonucleotides in concentrated (2.7 mol litre^{-1}) potassium phosphate occurs predominantly through purine bases.[177] Despite its obvious advantages over other methods, this method has only one disadvantage, *viz.*, the uncertainty of the binding site of nucleic acids. The use of an extended bisoxirane reagent ensures the introduction of a long uncharged hydrophilic arm between the ligand and the polymer surface, which is especially convenient for chromatography of large biomolecules. The affinity sorbents prepared on such supports are rather stable, since the ligand is attached through an ether bond, and the immobilisation is not accompanied by non-specific sorption of biopolymers.[15, 171] Immobilisation of biologically active ligands on mechanically stable silica gels containing epoxy groups allows preparation of matrices for high-performance affinity chromatography. It should be remembered, however, that some supports are unstable in alkaline media where such immobilisation takes place.

c. Supports containing *N*-hydroxysuccinimide groups

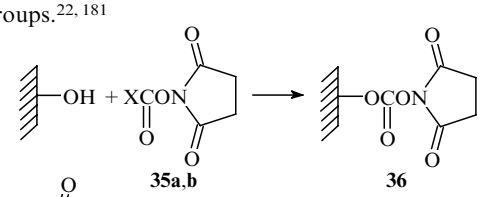
The idea to use *N*-hydroxysuccinimide esters as reactive groups for the formation of amide bonds with the primary amino groups of ligands was first formulated and realised in studies devoted to peptide synthesis.[178, 179] High solubility of *N*-hydroxysuccinimide in water and organic solvents makes it a convenient reagent for the activation of various polymeric surfaces.

The supports containing esterified *N*-hydroxysuccinimide **34** are obtained by treating the polymers containing terminal carboxy groups with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide (DCC) in dry solvents.[26, 180]

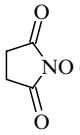


Bifunctional reagents, *e.g.*, *N*,*N*′-disuccinimidyl carbonate (**35a**) or *N*-succinimidyloxycarbonyl chloride (**35b**), are used for

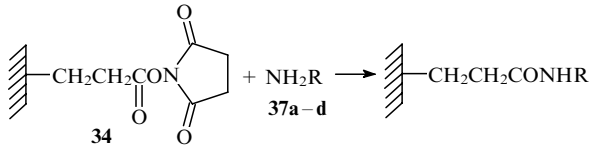
the activation of supports containing hydroxy or amino groups.[22, 181]



35a,b **36**

X =  NO (**a**), Cl (**b**).

The *N*-hydroxysuccinimide esters **34** and **36** react rapidly with oligonucleotides containing aminoalkyl groups at the 5′-terminal phosphate (**37a–d**) or in the heterocyclic bases in buffer solutions (pH 7.5–9.0) resulting in the formation of stable amide bonds.



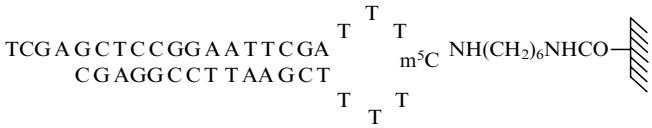
34 **37a–d**

R = $(CH_2)_6NH(pT)_{29}$ (**a**), $(CH_2)_2SS(CH_2)_2NH(pN)_{29}$ (**b**), $(CH_2)_{12}O(pCTT)_7$ (**c**), $(CH_2)_2O(pT)_{18}$ (**d**).

It was shown [65] that immobilisation of a 29-membered oligonucleotide occurs predominantly through the amino group of 6-aminohexyl linker attached as 5′-phosphoramidates (**37a**). Immobilisation through the cystaminyl linker (**37b**) is less efficient, while binding through the amino group of the heterocyclic bases proceeds to a smaller degree. Unfortunately, the capacity of the affinity sorbents thus obtained does not exceed 1 nmol g^{-1} at best, apparently, due to rapid hydrolysis of the activated ester **34**. The efficiency of immobilisation is higher in non-aqueous media where competitive hydrolysis is reduced to a minimum.

Hydrolysis of the reactive surface groups of the support **34** yields carboxy groups which confer ion-exchange properties on the affinity sorbent, while unconsumed groups of the activated support **36** are gradually hydrolysed to regenerate the starting groups.

An interesting approach to the immobilisation of double-stranded fragments of DNA on *N*-hydroxysuccinimide-activated Sepharose has been proposed.[31, 182] A 39-membered oligonucleotide was synthesised with a hairpin and a 1,6-diaminohexane unit was introduced into a heterocyclic base of the hairpin loop. The incorporated amino group was used for the attachment of the oligonucleotide to the polymer. Any double-stranded DNA fragments containing the corresponding sticky ends can be bound then to the immobilised oligonucleotide using DNA-ligase.



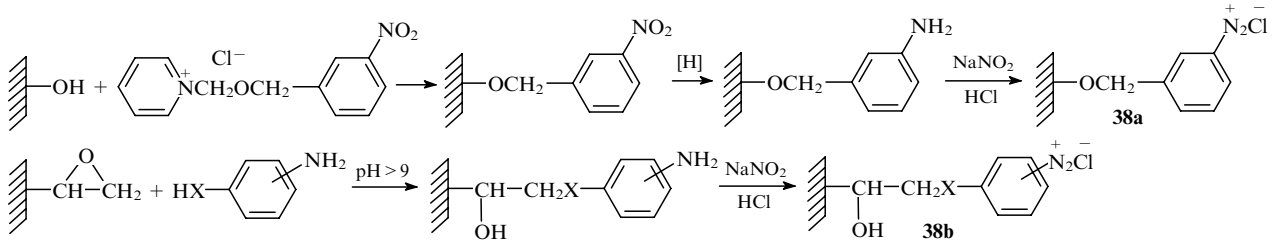
Supports with immobilised poly- and oligonucleotides were used in hybridisation analysis [21, 22, 183] and affinity chromatography.[7, 26] Thus the amino-containing oligonucleotide $(CTT)_7$ **37c** was immobilised on Sepharose and this sorbent was used for the isolation of plasmid pXL2563 containing GAA sites due to triplex formation.[7] Column chromatography on silica gel containing immobilised 5′-aminoethyloctadecathymidylate (**37d**) was successfully used for the separation of a mixture of adenylates $A_{12}-A_{18}$.[26]

d. Diazotised supports

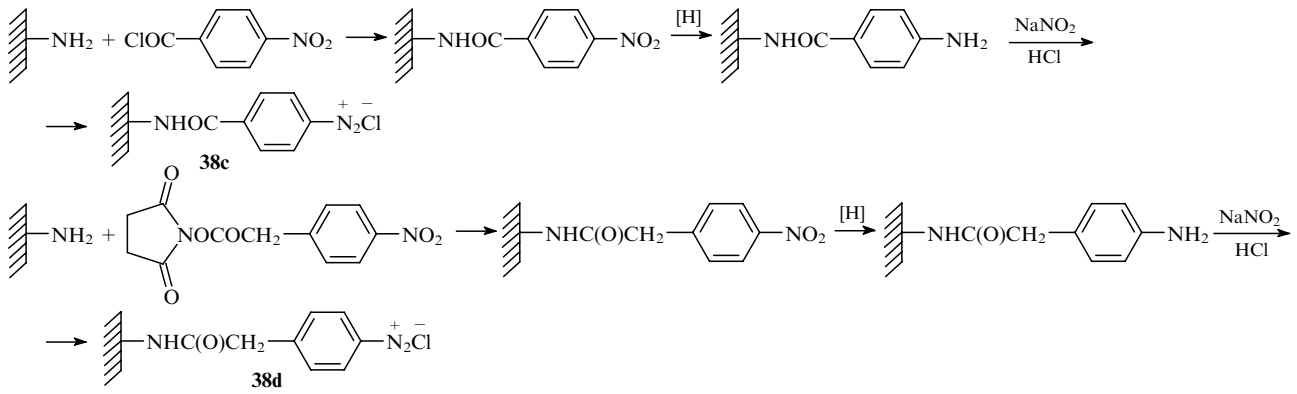
The azo coupling reaction has long attracted the attention of investigators engaged in immobilisation of nucleotides, since the introduction of aromatic amino groups into the support can be performed under mild conditions. Different approaches to the synthesis of polymers containing aromatic amino groups are shown in Scheme 1.[144, 184–186] Such supports are usually prepared by the reduction of the corresponding aromatic nitro compounds.[187] Diazotisation of aromatic amino groups is carried out with sodium nitrite in acidic media.

Immobilisation of native nucleic acids on diazotised supports, such as compounds **38a–d** (see Scheme 1), involves exclusively heterocyclic bases.[25, 67, 184] The introduction of spacer groups into oligonucleotides, *e.g.*, the introduction of phenol residues into 5′-phosphate groups, results in the predominant (up to 87%) immobilisation at this particular group.[188]

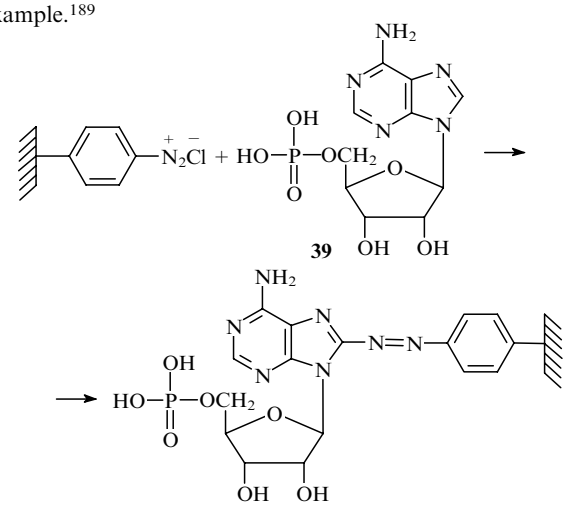
Scheme 1



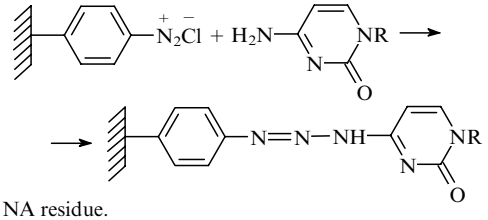
X = NH, S.



The azo coupling reaction consists in electrophilic substitution where the diazonium ion plays the role of an attacking agent. This reaction can be visually monitored by following colouration of the support. Immobilisation of 5′-AMP (**39**) at the C(8) atom of the heterocyclic base on agarose containing diazo groups is an example.[189]



Azo coupling can be accompanied by the reaction of diazonium salts with the amino groups of heterocyclic bases, which affords a diazoamino compound.[190]



R, NA residue.

In addition to reactions of diazonium salts with heterocyclic bases of nucleic acids, side reactions with water (to give phenols) and aromatic amino groups which are always present on the surface of such supports can also take place. If azo couping occurs at a slow rate, the formation of phenol and the cross-linking of the polymeric support can predominate.[185]

Aromatic diazo compounds are rather unstable, they decompose at elevated temperatures and upon illumination. Therefore, diazotised supports are used immediately after their synthesis. They can also be treated with $NaBF_4$ or $NaPF_6$ to give more stable tetrafluoroborates or hexafluorophosphates.[185]

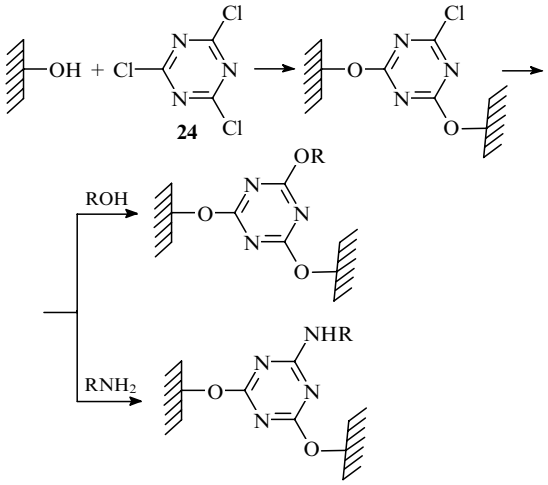
Denatured DNA and RNA immobilised by this method were used mostly in hybridisation analysis.[184, 185, 191] Good results were obtained with the use of supports **38b** (see Scheme 1) containing diazophenylsulfide groups.[140] Immobilised cyclic DNA was used as a template in an asymmetric polymerase reaction.[192]

The efficiency of immobilisation on diazotised supports is differently estimated by different authors, *viz*., from 795 μg of the nucleotide per 1 g of the support [146] to trace amounts.[25] Affinity sorbents prepared by azo coupling are usually unstable. Up to 50% of immobilised DNA is lost within the first 2–3 days at 45 °C irrespective of the nature of the matrix.[146] Low non-specific sorption of biopolymers on diazotised supports is their main advantage.

Cellulose or paper filters containing diazo groups are most commonly used for immobilisation of DNA; however, Sephacryl proved to be the best polymer for such immobilisation.[67] Magnetic particles were also tested, however, with little effect.[25]

e. Supports containing active halogen atoms

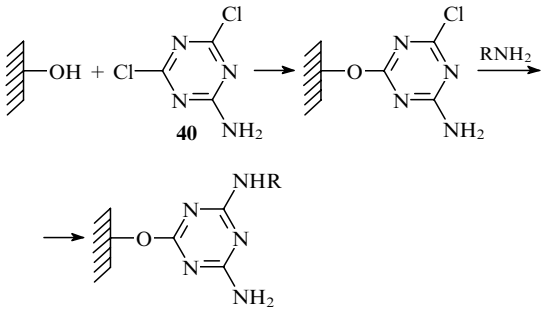
Activation of oligonucleotides by 2,4,6-trichloro-1,3,5-triazine (**24**) was considered in Section III.1.d. In this section, we shall consider the preparation of supports activated by 2,4,6-trichloro-1,3,5-triazine and their use for the immobilisation of ligands. The reactions of polynucleotides and NA with non-modified polysaccharides (cellulose and dextran) in the presence of 2,4,6-trichloro-1,3,5-triazine (**24**) [107] which permit consecutive substitution of chlorine atoms will also be discussed.



R, NA or oligonucleotide residue.

When the activation is carried out in an alkaline medium, two chlorine atoms react with the hydroxy groups of the supports resulting in the binding of triazine to the polymers. The third chlorine atom remains intact and can be substituted by the amino or hydroxy groups of the ligand to be attached.[193, 194] It was shown that immobilisation of DNA using 2,4,6-trichloro-1,3,5-triazine involves predominantly the amino groups of the heterocyclic bases.[107] As a result, the multipoint binding of DNA and oligonucleotides to the polymer makes the ligand less accessible for the interaction with the complementary polynucleotides in solution. Immobilisation of oligonucleotides bearing an amino group-containing linker at the 5′-end occurs selectively through the introduced amino group.[107, 194] However, even in this case the yields of the addition products are rather low due to the low accessibility of the chlorine atom in the triazine-containing support.

In order to increase the efficiency and selectivity of the method, it was suggested to activate the supports by 2-amino-4,6-dichloro-1,3,5-triazine (**40**).[142, 193]



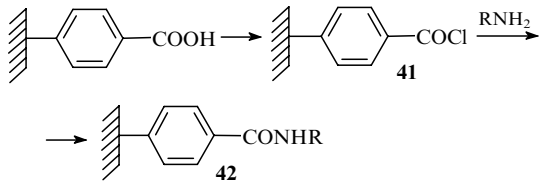
R, oligonucleotide residue.

The presence of an amino group in the triazine **40** decreases the reactivity of one of the chlorine atoms with respect to nucleophilic attack and thus prevents cross-linking of the polymer. The remaining chlorine atom reacts exclusively with strong nucleophiles, *e.g*., with the aliphatic amino group of the spacer introduced into an oligonucleotide.[193]

Triazine-activated polymers are fairly stable at elevated temperatures and can be stored for several weeks at 4 °C. The use of matrices with immobilised DNA or poly(U) made it possible to isolate both proteins and the complementary chains of NA.[193] However, application of this method is restricted by the high toxicity of trichlorotriazine.

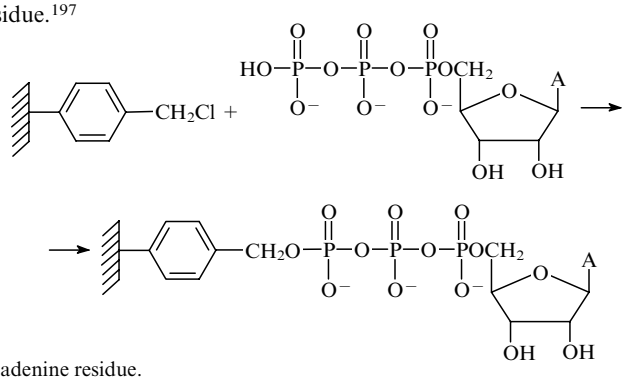
The carboxy groups of polyethylene terephthalate films can easily be converted into acid halide groups upon treatment with PCl_3, PCl_5, $POCl_3$, $SOCl_2$ or $(COCl)_2$ in the presence of DMF.

The polymers **41** containing acid halide groups react with the nucleophilic groups of the ligands. Immobilisation carried out under anhydrous conditions involves predominantly primary amino groups of the spacer incorporated into the 5′-terminal phosphate groups of oligonucleotides.[195] The stability of the sorbents **42** is provided by the stability of the amide bond formed. The films containing immobilised oligonucleotides obtained by this method were successfully used in hybridisation analysis.[196]



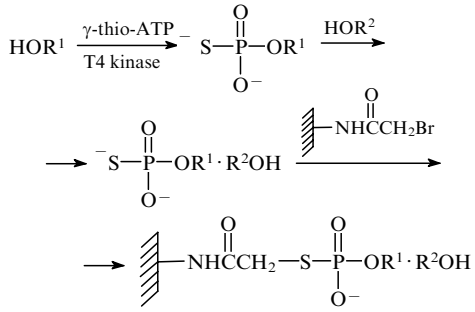
R, NA or oligonucleotide residue.

A chloromethylated styrene–divinylbenzene copolymer can also be used for the immobilisation of ligands. This approach was used for the attachment of 5′-ATP through the triphosphate residue.[197]



A, adenine residue.

Halogenoacetyl agarose has become especially popular among commercially available supports containing active halogen atoms (see Table 2). This sorbent is resistant to hydrolysis; therefore, the immobilisation procedure can be carried out in aqueous buffers. The best results were obtained in the case of immobilisation of oligonucleotides containing thiol groups. To prevent attachment at heterocyclic bases, the SH-containing oligonucleotide obtained with the use of T4 kinase and γ-thiol-ATP was hybridised with the complementary chain.[137] The duplex formed was attached to bromoacetyl agarose. The immobilisation occurred exclusively at the 5′-terminal thiolphosphate group of the oligonucleotide. If required, the complementary chain could be removed.

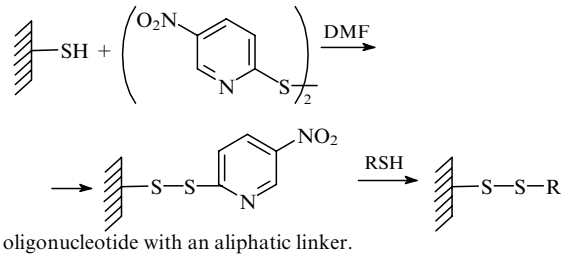


R^1,R^2, complementary oligonucleotide residues.

f. Activated supports for the immobilisation of SH-containing oligonucleotides

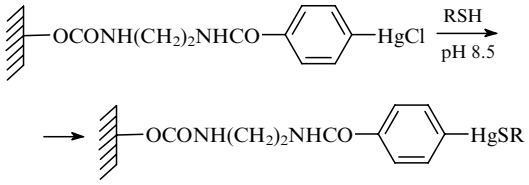
Immobilisation of NA and their fragments containing thiol groups on supports containing epoxy groups or active halogen atoms[134] has been described in Sections III.2.b and III.2.e, respectively. SH-containing ligands can also be immobilised using other methods.

Oligonucleotides containing thiol groups can selectively be immobilised on solid supports containing thiol groups owing to the formation of disulfide bonds. The supports are pretreated with *p*-chloromercuribenzoate, di(3-ethoxycarbonyl-4-nitrophenyl) disulfide, 2,2′-dipyridyl disulfide or 2,2′-dithiobis(5-nitropyridine).[90,144]



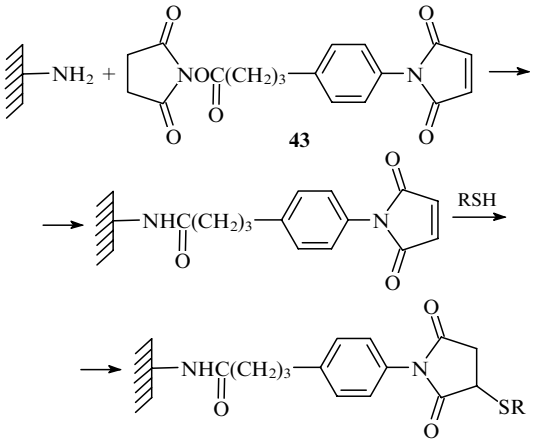
R, oligonucleotide with an aliphatic linker.

High thiophilicity of mercury ions allows the attachment of SH-containing oligonucleotides to modified polymers (*e.g.*, to agarose) containing HgCl groups.[90]

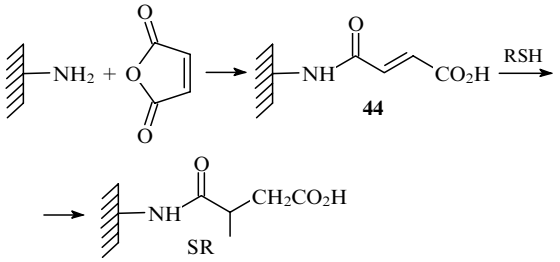


R, oligonucleotide with an aliphatic linker.

The reaction of the thiol groups with the maleimide residues present on the polymer surface is yet another approach to the immobilisation of SH-containing oligonucleotides. Maleimide can be incorporated into virtually all materials containing primary amino groups using succinimidyl 4-(maleimidophenyl)butyrate (**43**) or maleic anhydride.[11,16,136]



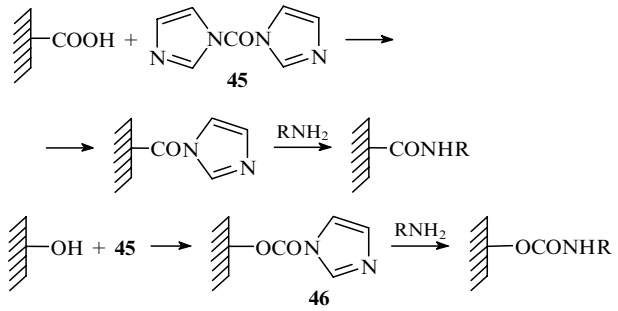
The nucleophilic addition of SH-containing oligonucleotides to the double bonds of the support **44** in a buffer solution occurs rapidly (2 h at 4 °C) resulting in the formation of a stable bond between the support and an oligonucleotide.



R, oligonucleotide residue.

g. Activation of supports by carbonyldiimidazole

Hydroxy and carboxy groups of supports can easily be activated by *N*,*N*′-carbonyldiimidazole (**45**). This reagent earlier proposed for peptide synthesis [198] is now successfully used for the immobilisation of ligands containing primary amino groups.[199, 200]



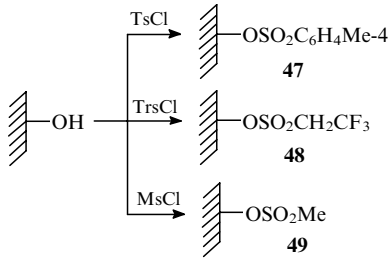
R, oligonucleotide residue.

The activation of polymers is performed in non-aqueous media; the optimum conditions for the immobilisation of ligands must be selected for each particular case. The high efficiency of the ligand attachment in dry solvents is due to the lack of competitive hydrolysis. Affinity matrices prepared by this method are rather stable, since the amide bond formed between the support and the ligand is very strong. The support **46** is stable in dry solvents for several years. The half-life time of the hydrolysis of imidazolyl carbamate **46** is several hours. Thus activated agarose loses completely its activity in aqueous buffers only after 30 h at pH 8.5–9.0.[200] Following elimination of CO_2 and imidazole, the support **46** is converted into the original hydroxyl-containing carrier devoid of charged groups, which excludes the manifestation of ion-exchange properties of the support and non-specific sorption of biomolecules. It is noteworthy that the half-lifetime of the hydrolysis of cyanogen bromide- or hydroxysuccinimide-activated supports is several minutes.

However, despite the advantages of the above-described imidazole-containing supports the immobilisation of, *e.g.*, 6-aminohexylphosphoryl-dCTP and 5′-aminoalkyloligonucleotides, occurs with low yields.[3, 201] This method has not found wide application for the immobilisation of NA and its fragments.

h. Activation of supports by sulfonyl chlorides

Organic sulfonyl chlorides, such as *p*-toluenesulfonyl chloride (TsCl), 2,2,2-trifluoroethanesulfonyl chloride (TrsCl) and methanesulfonyl chloride (MsCl), are potent acylating agents, which convert hydroxy groups of a carrier into reactive sulfonates to afford the supports **47**–**49**.[202–204]



TsCl = $ClSO_2C_6H_4Me$-4, TrsCl = $ClSO_2CH_2CF_3$, MsCl = $ClSO_2Me$.

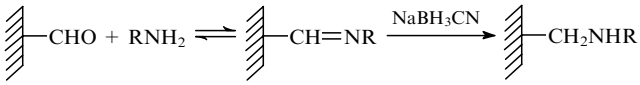
Immobilisation of oligonucleotides on the supports **48** [205] and **49** [29] occurs exclusively through the amino group of a spacer attached to the 5′- or 3′-end of the oligonucleotide. In the absence of aminospacers, immobilisation through heterocyclic bases and terminal phosphate groups of oligonucleotides proceeds with lower yields. Immobilisation on the support **49** occurs with low yields,[29] whereas polymers **48** are used for the preparation of affinity sorbents with high capacities; these are used for purification of DNA-dependent proteins.[205] The sulfonate residues are good leaving groups and the reactivities of the activated supports depend on the nature of the sulfonate and decrease in the following order: Trs > Ts > Ms. Thus, the support **49** is the most stable and the least reactive. The supports **47**–**49** are stable in non-aqueous media and can be stored for several weeks at 4 °C in aqueous HCl (1 mmol litre^{-1}) without any loss in their activities. Immobilisation of NA containing thiol or primary amino groups on sulfonyl chloride-activated supports occurs as the nucleophilic substitution of the sulfonate residue with the formation of a stable bond between the nucleophile and the carbon atom of the original support. The attachment of the ligand can be carried out in both aqueous and organic media. Immobilisation on the more reactive polymers **48** is more efficient at neutral pH values at 4 °C, whereas that on the carriers **47** is better performed at pH 9–11.[204]

i. Supports containing aldehyde groups (reductive amination)

Immobilisation of NA and their fragments by reductive amination can be carried out using two approaches. The first of them consists in the introduction of the aldehyde groups into NA and their subsequent immobilisation on supports containing hydrazide or primary aliphatic amino groups (see Section III.1.e). The second approach includes the incorporation of aldehyde groups into supports and their subsequent application for the immobilisation of NA through the amino groups of heterocyclic bases or through introduced spacer groups. The latter version is considered in this section.

Aldehyde groups can be introduced into supports by different methods. Polysaccharide supports containing diol groups are oxidised by sodium periodate to give the modified polymer **50** with high content of the aldehyde groups. The supports devoid of hydroxy groups are modified by glutaraldehyde (**51**), glycidol (**52**), an oligomer containing aldehyde groups (**53**) or compounds which are further oxidised by periodate. Some examples of preparation of polymers of virtually any type containing aldehyde groups are shown in Scheme 2.

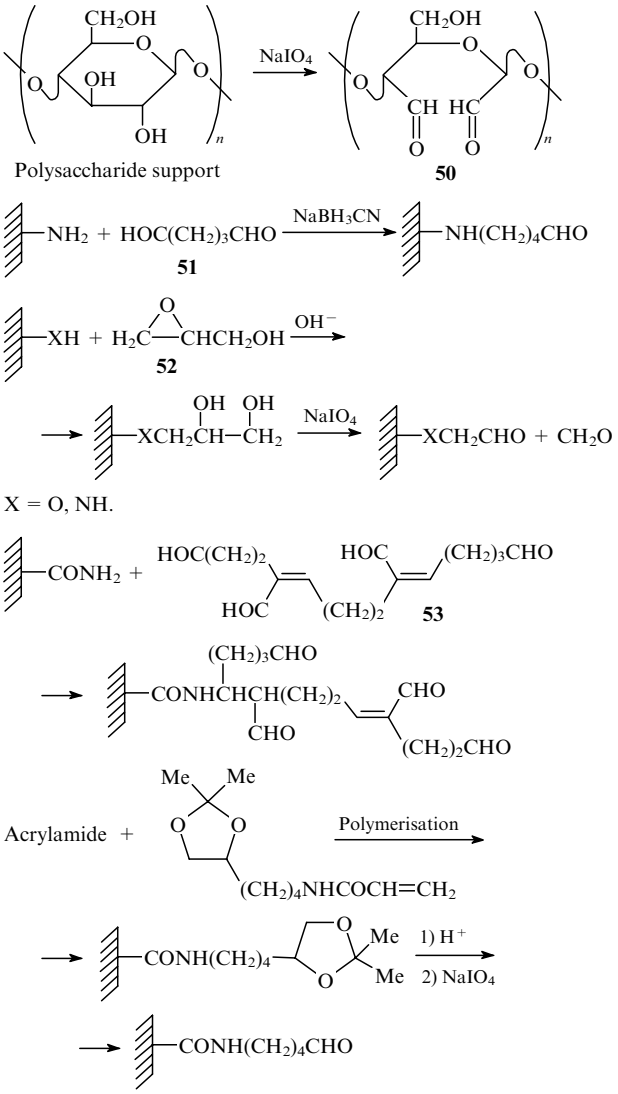
The aldehyde groups of the polymer react readily with ligands containing primary amino groups to give Schiff's bases. The latter are further reduced by $NaBH_4$, $NaBH_3CN$ or the pyridine–borane complex ($Py \cdot BH_3$). Unlike sodium borohydride, the last two reagents rapidly reduce the Schiff's bases, but not the aldehyde groups.[29, 206] It should also be noted that $NaBH_4$ is decomposed in acidic media, whereas $NaBH_3CN$ functions effectively at pH 4–10. It was recommended to replace highly toxic $NaBH_3CN$ by the $Py \cdot BH_3$ complex.[29]



Oligonucleotides with incorporated aminospacers are immobilised in good yields on supports containing aldehyde groups to form strong bonds.[11, 12, 29, 30] However, this is accompanied by the addition of oligonucleotides through the amino groups of the heterocyclic bases. Especially strong non-specific immobilisation was observed in the case of supports activated by glutaraldehyde (**51**).[29]

The glutaraldehyde **51** is used to prepare affinity sorbents with a high content of NA. This reagent brings about cross-linking of the polysaccharides with nucleotides and NA (DNA, RNA [207], NAD [208]) through primary amino groups of the modified carriers (aminoethylcellulose,[207] aminohexylsepharose [208]) and the heterocyclic bases. High concentrations of the ligand on the carrier results from additional intramolecular cross-linking of the ligand, which is often a drawback, especially in affinity chromatography.

Scheme 2



Polysaccharide support

50

51

52

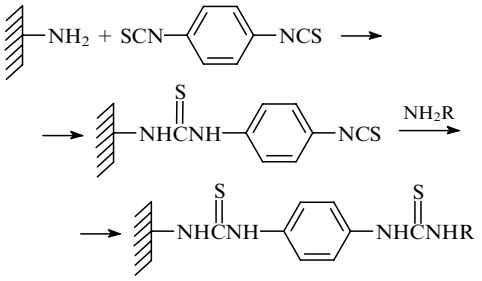
X = O, NH.

53

Acrylamide +

j. Supports bearing isothiocyanate groups

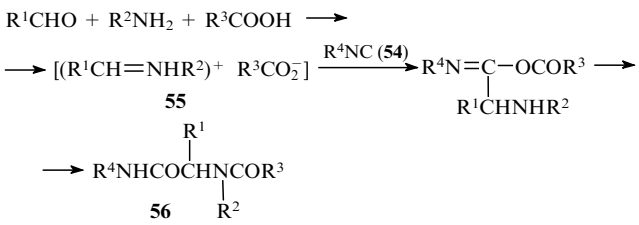
The isothiocyanate group is yet another functional group which rapidly reacts with aliphatic primary amino groups to give stable thiourea derivatives. 1,4-Phenylene diisocyanate can be used for the activation of virtually all polymers containing primary amino groups.[17, 19, 22] Immobilisation of oligonucleotides on isothiocyanate-containing supports occurs with good yields[17, 19] and is not accompanied by immobilisation of non-modified oligonucleotides.[19, 22] The remaining isothiocyanate groups of the activated carrier undergo gradual hydrolysis to amino groups, which imparts ion-exchange properties to the affinity matrices.



3-Isothiocyanatopropyltriethoxysilane [209] or 1-(3-trimethoxysilylpropyl)-3-(4-isothiocyanatophenyl)thiourea [19] can be used for the modification of glass surfaces. This one-step procedure is used for the preparation of glasses bearing reactive isothiocyanate groups.

k. The four-component condensation

The Ugi reaction is a multicomponent condensation leading to *N*-alkylamides under mild conditions [171, 210–212] and involves α-addition of electrophilic compounds containing C=N bonds to isocyanides **54**. The immonium salt **55** formed in the reaction of a carboxylic acid with an amine and an aldehyde or a ketone is added to isocyanide. Intramolecular acylation affords the final product **56**.



55

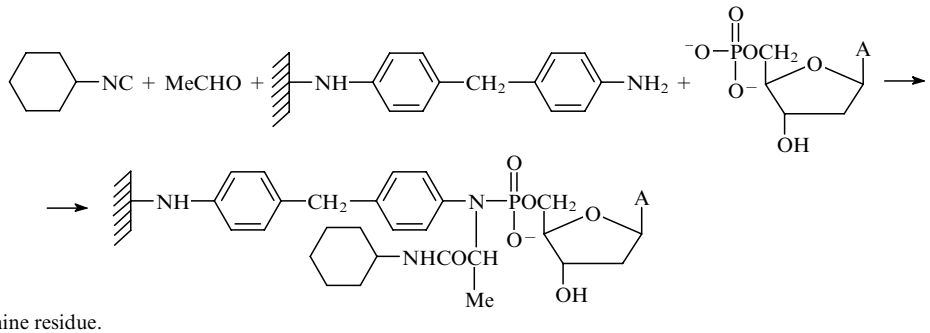
56

The three soluble reactants may be varied depending on the nature of the functional groups (isocyanide,[201] carboxy, aldehyde or amino groups [212]) present on the support surface.

The reaction of 5′-AMP with Sepharose modified by 4,4′-diaminodiphenylmethane in the presence of cyclohexyl isocyanide and acetaldehyde is an example of the Ugi reaction employed for the preparation of modified supports.[171, 212, 213] In this case, the role of an anion of the immonium salt **55** is played by the phosphoric acid residue of 5′-AMP and not by the carboxy group (Scheme 3).

The best results (39 mg g^{-1}) were obtained in the immobilisation of RNA on Sepharose containing amino groups.[212] However, this method has not found wide application due to the side reactions between the functional groups of the nucleotides and the components of this complex system.

Scheme 3



A, adenine residue.

IV. Immobilisation of nucleic acids in the course of solid-phase synthesis

The development of automated methods for oligonucleotide synthesis offered new opportunities for the preparation of affinity matrices bearing covalently bound oligonucleotides. The carriers on which oligonucleotides are synthesised can be used as affinity sorbents. This obviates the necessity of removal of the nucleotide material from the carrier and its further immobilisation on the support. However, one essential step, *viz*., purification of the newly synthesised oligonucleotide and the estimation of the quality of the immobilised material, is lost.

The last step in any solid-phase oligonucleotide synthesis includes the removal of protective groups from the heterocyclic bases and splitting of the nucleotide material from the support by treatment with concentrated ammonia. Elimination of the nucleotide material under deblocking conditions can be excluded if oligodeoxythymidylates [214] devoid of protective groups are synthesised or if supports are used containing primary aliphatic amino [8, 22, 215] or hydroxy [1, 216–218] anchor groups or the hydroxy groups of preliminarily attached nucleosides.[31, 219] Oligonucleotide synthesis on such supports results in the formation of an ether bond resistant to ammonia. Monomers containing non-conventional protective groups of heterocyclic bases are also used in the synthesis of affinity sorbents; their removal requires milder conditions,[220] which do not cause the destruction of the ester bond between the support and the nucleotide residue.

In addition to macroporous glass [8, 31, 214, 216, 217, 220] and glass slides,[22] polyacrylmorpholide,[31] polypropylene,[215] silica gel [31] and Teflon copolymers [216] are used as supports for oligonucleotide synthesis. The affinity matrices based on them manifest good hybridisation properties [8, 215, 217] and can be used for the isolation of mRNA [214] and DNA-specific proteins.[216]

The advantages and disadvantages of different supports in the synthesis of oligonucleotide libraries (combinatorial synthesis) have been reviewed in detail.[218] TentaGel (polyoxyethylene-coated polystyrene beads, Rapp Polymere GmbH) and Merck-Gel [partially hydrolysed poly(vinyl acetate), Merck KgaA] were recognised as the best granulated carriers. Their capacities with respect to the first fragment (nucleoside) are 120 and 700 μmol g^{-1}, respectively (*cf*. 30–50 μmol g^{-1} for conventional supports); the elongation of the oligonucleotide chain occurs also with high yields.

At present, new procedures and equipment for oligonucleotide synthesis on films and glass slides used as DNA chips and DNA sensors are being developed (see Section VII.2.b).

V. Miscellaneous methods of immobilisation of nucleic acids

Physical and physicochemical methods have been used for the immobilisation of NA earlier than other methods. Adsorption, UV irradiation and mechanical incorporation into different gels are especially popular nowadays.

Adsorption of DNA on cellulose is a very simple and rather widespread procedure which consists in freeze-drying [221–223] or conventional drying [222, 224] of cellulose impregnated with solutions of NA. The DNA-cellulose thus prepared is successfully used for purification of DNA-dependent proteins. However, such DNA-cellulose is not always suitable for hybridisation of NA because of instability of the nucleotide material at elevated temperatures and its easy desorption in buffers of low ionic strength. Nevertheless, non-covalent sorption of DNA fragments on nitrocellulose filters is a very popular procedure for the preparation of samples for hybridisation analysis [225] despite its obvious disadvantages, such as the loss of the nucleotide material during multistage hybridisation assays, fragility of cellulose after vacuum treatment, *etc*.

The development of polymerase chain reaction (PCR) and the advent of new methods of hybridisation assay brought forth the need for rapid immobilisation of NA fragments on the surface of microplates. Adsorption of oligonucleotides on such surfaces is carried out in the presence of high salt concentrations (NaCl, tetramethylammonium chloride) and/or cationic detergents (cetyltrimethylammonium bromide, dimethyloctylamine hydrochloride).[88, 226–229] Presumably, in this case immobilisation occurs exclusively by virtue of hydrophobic interactions between the oligonucleotides and the support surface.[88] It was noted [22] that adsorption is selective as regards surfaces which possess hydrophilic properties.[226] Although the mechanism of such immobilisation is not completely understood, the samples prepared by this method are widely used in hybridisation assays.[88, 227, 228]

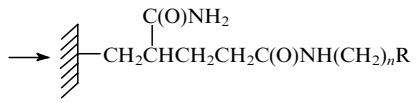
Yet another procedure consists in the application of polyelectrolyte films onto glass surfaces and the sorption of DNA or polynucleotides occurs owing to ion-exchange interactions.[230, 231]

Nucleic acids and their fragments can be immobilised on different supports upon irradiation with UV light.[155, 222, 223, 232–235] Side reactions occurring in the ligands are not significant and do not prevent the application of these systems in affinity chromatography of proteins and enzymes.[222, 232, 234] The use of specially prepared photosensitive supports, *e.g*., cellulose [236] or microporous Nylon 6 [237] containing aryl azide residues, increases significantly the maximum capacity and efficiency of photoimmobilisation of NA. The sensitivity of hybridisation assays with these matrices is 2- to 4-fold higher than that obtained with matrices prepared by conventional sorption on nitrocellulose. This may be ascribed to mild immobilisation conditions which do not affect the structure of NA.

Nucleic acids or their fragments can be immobilised on polyacrylamide,[238, 239] agar [240] or on glass slides bearing the acrylamide groups,[20, 241] which allows the preparation of sorbents with high content of the nucleotide material. The advantage of this method is that the immobilisation can be carried out on virtually any surface.[241, 242]

To ensure higher selectivity of immobilisation, copolymerisation is carried out with oligonucleotides functionalised with groups containing multiple bonds (*e.g*., pyrrolyl,[242] allyl [20] or acrylamide [241]).

$$NH_2C(O)CH{=}CH_2 + CH_2{=}CHC(O)NH(CH_2)_nR \xrightarrow{\text{Polymerisation}}$$



R, oligonucleotide residue.

VI. Immobilisation of nucleic acids using enzymes and avidin–biotin interactions

Recently, streptavidin- or avidin-containing sorbents have gained wide acceptance for the attachment of biotin-modified DNA or oligonucleotides. In these matrices, immobilisation of oligonucleotides is achieved due to the formation of a stable avidin–biotin complex ($K_d \approx 10^{-15}$ mol litre^{-1}) rather than a covalent bond. Such sorbents are easily and rapidly prepared by mechanical mixing of a streptavidin carrier and a biotin-modified oligonucleotide and used for the isolation of proteins and enzymes,[35, 36, 243–246] DNA sequencing [34, 247] and hybridisation assays.[248, 249] However, these sorbents possess a number of disadvantages, such as non-specific sorption of proteins which is more pronounced in the case of avidin-containing matrices,[250, 251] than in the case of streptavidin supports.[252] The remaining avidin residues are blocked by bovine serum albumin or methyl mannoside;[253] buffers of high ionic strength are used for chromatography.

Short and homogeneous oligonucleotides are more accessible and can be immobilised more easily than long NA fragments. The sorbents containing oligo(dT) [especially oligo$(dT)_{25}$-Dynabeads] and oligo(dA) have now become especially popular.[254–256] Immo-

bilisation of large fragments of NA on these or any other supports containing short oligonucleotides (10–20 nucleotide residues) can be carried out enzymically using ligase, by attaching specially synthesised or isolated long fragments of NA,[24, 257] or using DNA-polymerase, by completing the mRNA [258, 259] or the DNA template [260] which forms complexes with the short oligonucleotide on the polymer.

VII. The use of matrices containing nucleic acids

1. Affinity chromatography

The solution of the key problems of molecular biology and biochemistry related to the structure and interactions between biopolymers demands the use of special techniques allowing the isolation of individual biopolymers from complex multicomponent mixtures. Thus proteins, enzymes, NA, oligonucleotides and their analogues are necessary in a pure state for the studies of their structures as well as for protein-NA, DNA-DNA and RNA-DNA recognitions. These biomolecules can be isolated by affinity chromatography on sorbents containing immobilised NA or oligonucleotides. This method is based on the ability of ligands immobilised on insoluble supports to form highly specific complexes with macromolecules, which dissociate under definite conditions (*e.g.*, either upon change in the ionic strength or pH of the eluent or upon increase in temperature).

a. Isolation of individual nucleic acids and their fragments

The principle of affinity chromatography of NA was first applied to the isolation of oligo- and polynucleotides having homogeneous sequences. The possibility of fractionation of oligonucleotides on supports containing immobilised polynucleotides has been noted in the early papers.[60, 61, 261] A series of papers devoted to affinity chromatography of poly(A)-mRNA,[214, 254, 262, 263] NA or oligonucleotides containing homogeneous sequences were published in the late 1980's–early 1990's owing to the development of methods of synthesis of oligonucleotides and the accessibility of novel carriers.[26, 264] However, this method was not successful for the isolation of individual oligonucleotides or longer sequences devoid of homogeneous inserts. Present-day studies widely employ commercial supports carrying immobilised homogeneous oligonucleotides, most commonly, oligo(T) supports. The use of durable materials, such as silica gel, glass and some methacrylate-based copolymers as supports allows high-performance affinity chromatography and complete separation of oligonucleotides differing in length by one base.[105]

Affinity chromatography of heterogeneous oligonucleotides and NA has become possible following development of efficient methods for immobilisation of heterogeneous oligonucleotides on insoluble supports. Matrices containing relatively short (10–15-mers) immobilised oligonucleotide fragments complementary to a region of an oligonucleotide to be purified have been used for the isolation of extended (more than 30-mers) synthetic oligonucleotides which can hardly be purified by conventional chromatographic methods.[102, 104] This approach was applied for the isolation of a synthetic 34-membered oligonucleotide representing a deoxy copy of an RNA fragment of the Russian tick-borne encephalitis virus on LiChrosorb-NH_2 bearing an immobilised 17-membered oligonucleotide complementary to the 5′-end of the 34-membered fragment (Fig. 2).[102]

Apart from synthetic oligonucleotides, individual NA and DNA fragments obtained upon scission of DNA with restriction enzymes can be isolated by affinity chromatography from complex mixtures (see Fig. 2).[102, 104] This approach was used to obtain highly enriched $tRNA^{Phe}$ from human placenta [103] utilising two successive affinity chromatography steps on matrices containing immobilised oligonucleotides complementary to the CCA-end of tRNA (the first step) and to a site in the anti-codon loop of $tRNA^{Phe}$ (the second step).

Both single- and double-stranded NA can be isolated from cell extracts on sorbents carrying immobilised specific oligonucleotides. In the latter case, oligonucleotides should be able to form triplexes with definite regions of NA, as can be exemplified by purification of the DNA plasmids pTS2 (Ref. 265) and pXL2563 (Ref. 7) from cell extracts.

b. Separation of diastereomeric mixtures of non-ionic oligonucleotide analogues

Considerable interest in oligonucleotide analogues containing modified internucleotide phosphate groups, *viz.*, methylphosphonates and phosphotriesters, can be explained by their physicochemical and biological properties, *e.g.*, resistance to nucleases, sorption on cell membranes, penetration inside the cell and ability to form complexes with the complementary regions of NA. Since these analogues contain an asymmetric internucleotide phosphorus atom, they represent mixtures consisting of a large number of diastereomers which differ in the stabilities of the complementary complexes formed. Individual diastereomers are necessary for detailed investigations of physicochemical properties and biological behaviour of oligonucleotide analogues in cellular systems.

For the first time, chromatographic separation of diastereomers of nonathymidilyluridine octaethyl ester into fractions was carried out on a column with poly(A) cyanogen bromide-activated Sepharose.[266] However, individual diastereomers of non-ionic analogues of oligonucleotides could be obtained only by high-performance affinity chromatography.

The silica gel-based sorbent LiChrosorb bearing the octanucleotide pTGTTTGGC immobilised through the 5′-terminal phosphate group was used for the isolation of individual diastereomers of the heterogeneous octanucleotide GCCAAACA ethyl esters at a constant column temperature.[105] This allowed separa-

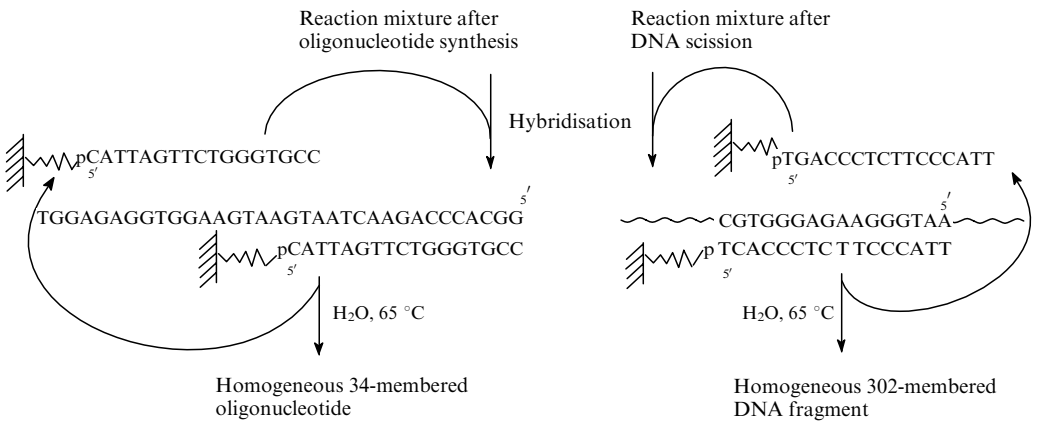


Figure 2. Affinity chromatography.

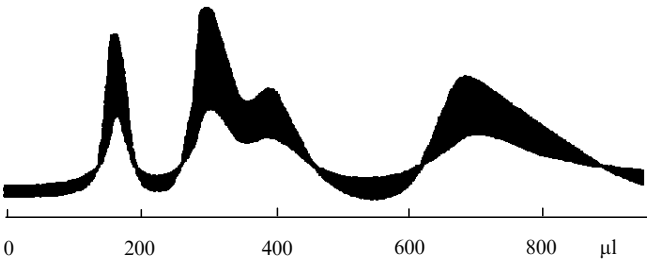


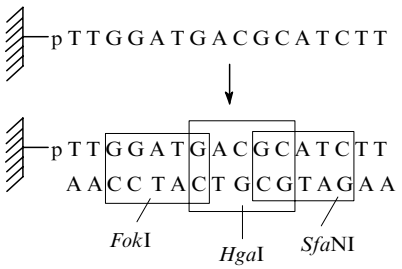
Figure 3. A chromatographic separation profile of a mixture of four diastereomers of the octanucleotide GpCpCpAp_{OEt}Ap_{OEt}ApCpA ethyl esters on an affinity sorbent (LiChrosorb) containing pTGTTTGGC immobilised through the 5′-terminal phosphate group.[105]

tion of stereoisomers of the octanucleotide GCCAAACA containing one, two or three phosphotriester groups (Fig. 3). The same affinity sorbent was successfully used to obtain individual diastereomers of the heptanucleoside methylphosphonate $Cp_{Me}Cp_{Me}Ap_{Me}$ $Ap_{Me}Ap_{Me}Cp_{Me}A$; the absolute configurations of these diastereomers have been established.[267]

c. Isolation of NA-dependent enzymes

At present, affinity chromatography on cellulose and Sepharose containing calf thymus DNA has become a routine procedure for obtaining pure preparations of NA-dependent proteins which are non-specific as regards the nucleotide sequence. Purification of site-specific proteins is carried out on sorbents with immobilised oligonucleotides having the corresponding recognition sites. Most of the proteins isolated represent transcription and replication protein factors (for a comprehensive description, see the reviews [181, 268, 269]). The list given in these reference can be supplemented by several other papers [36, 137, 270, 271] describing the methods for the preparation of affinity sorbents used for the purification of these proteins. Affinity chromatography was also used for the isolation of some receptors.[62, 168, 252]

Isolation of site-specific restrictases is of the greatest interest, since affinity matrices in this particular case should be resistant to enzymes to be isolated. The restriction endonucleases *Eco*RI [272] and *Sph*I [273] hydrolyse DNA only in the presence of Mg^{2+} ions, whereas in the absence of Mg^{2+} ions they bind to the site-bearing DNA. This property is used for their isolation. For the isolation of IIS-type restrictases (*Fok*I, *Hga*I, *Sfa*NI), which hydrolyse DNA at a site other than the recognition site, a duplex which incorporates the recognition sites for these enzymes but contains no sites necessary for DNA hydrolysis has been synthesised.[101] The structure of the duplex is given below (the corresponding recognition sites are enclosed in boxes). One of the oligonucleotides of the duplex was covalently linked to the methacrylate-based polymeric support.



Sorbents containing immobilised oligonucleotide duplexes, which, in turn, contain non-canonical base pairs, are used for the isolation of DNA repair enzymes responsible for mismatch correction. Thus thymine-DNA glycosylase [274, 275] was isolated on an affinity DNA sorbent containing a dG : U mismatch, however, in only trace amounts. Highly purified biologically active mismatch-specific DNA glycosylases (MutM and MutY) responsible for the removal of the oxidised form of guanine from DNA as well as for mismatch correction were isolated by affinity chromatography.[106] This study utilised a support (Toyopearl-AF) carrying an immobilised oligonucleotide duplex containing the non-canonical pairs $N8^{oxo}$-dC or rA-dG (where $N8^{oxo}$ is 8-oxonebularine, an analogue of the oxidised form of guanine).

Affinity chromatography on oligonucleotide-containing sorbents allows both the isolation of NA-dependent proteins and studies of their interactions with immobilised NA fragments.[106, 276]

2. Hybridisation assay

Molecular hybridisation of NA is based on the fundamental property of complementary chains of NA to form stable duplexes. This method allows rapid testing of mutated viral NA and viroids and is distinguished by high specificity, sensitivity and reproducibility. The main stages of the hybridisation analysis have been described in detail.[277–282]

The sensitivity of this method is determined by the choice of procedures for the detection of oligonucleotide probes carrying radioactive ([^{32}P]), fluorescent and other labels.

The selection of a support and the procedure for the immobilisation of NA or oligonucleotides is the key problem in any solid-phase hybridisation assay, which is determined by a specific goal in each particular case.

Dot-blot hybridisation includes immobilisation of a denatured portion of total NA to be tested on nitrocellulose.[283–289] Polystyrene immunological microwell plates are employed for the automation of serial tests of clinical samples.[290, 291] Immobilisation of NA targets or specific cloned fragments is carried out by physicochemical methods, *e.g.*, by sintering at 80 °C or by UV irradiation.

Blot-hybridisation in which a NA preparation is hydrolysed by restriction enzymes subjected to fractionation (*e.g.,* separation according to the molecular mass) by gel electrophoresis and further transferred to a support from the gel is a more efficient method of analysis. After immobilisation on the support, NA are hybridised with labelled probes (the so-called Southern blot DNA assay) [225] and Northern blot (RNA assay).[292] Currently, the Southern blot is used for the detection of PCR products and determination of the reaction specificity.[293–295] Nitrocellulose filters [225, 292, 296, 297] and nylon membranes [298, 299] (less frequently, *p*-azidobenzoylcellulose [236]) or dried gels following fractionation of NA fragments [300] are commonly used for the fixation of the nucleotide material. Sandwich blot is a promising approach to the analysis of NA in non-purified specimens containing proteins, polysaccharides and other components.[301] This method is based on the use of two different oligo- or polynucleotide fragments complementary to different sites of the NA to be assayed. One of these fragments is immobilised on a support, whereas the other is soluble and carries a reporter group (*e.g.*, a radiolabel, a biotin residue, *etc.*). After hybridisation and removal of admixtures and the excess of the second (labelled) probe, the NA that has formed a complementary complex with the nucleotide immobilised on the support is detected by the label bound. A vast number of solid supports and immobilisation procedures have been used for sandwich blot. Initially, nitrocellulose filters have been used,[302, 303] which were replaced by mechanically stronger nylon membranes [304] and more handy polypropylene and polystyrene immunological microwell plates.[32, 305–307] Hybridisation of NA with oligonucleotide probes immobilised on the surface of microgranulated supports is a way of acceleration of the hybridisation. Different versions of this approach using Sephacryl,[67] polyacrylamide,[28] nylon [27] and polystyrene beads [304, 308, 309] and magnetic microparticles [25, 219, 304, 310] have been described. The use of magnetic microparticles has an additional advantage, since they can easily be concentrated from suspensions, which significantly simplifies manipulations with samples.

'Reverse dot blot' [48] implies the introduction of a label into the NA or its fragment obtained by PCR. After hybridisation of a labelled NA with an oligonucleotide immobilised on a solid

support, the complementary hybrid formed is detected by virtue of the label bound to the support. Nylon [48, 66, 83, 311] and poly(ethylene terephthalate) [195, 312, 313] membranes, immunological microwell plates [314, 315] and glass [17, 316] have been used as supports for the reverse dot blot. The use of cover glasses in hybridisation assays is especially convenient, since glass is readily available, inexpensive and has a well-studied surface which is easy to modify and is suitable for fluorescent detection. Immobilisation of oligonucleotides is usually carried out by photochemical or various chemical methods.

3. DNA chips and DNA sensors

The development of effective methods for the immobilisation of oligonucleotides as well as array-based mapping methods have led to the design of microchips with immobilised oligonucleotide sets (arrays). Such DNA chips which represent slides (usually, glass slides) containing an immense variety of immobilised oligonucleotides (tens of thousands of DNA fragments) find ever growing application in molecular biology, medicine, biotechnology, *etc.*

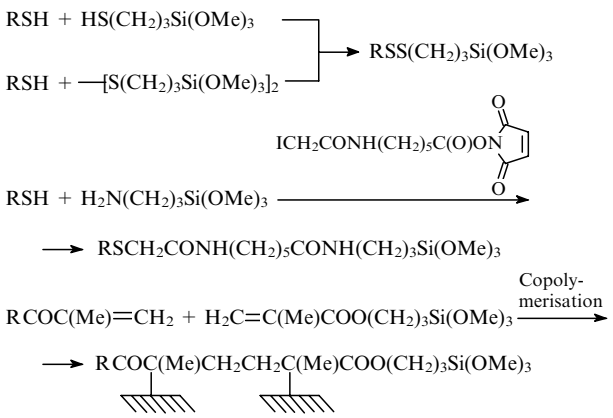
Initially, microchips were proposed for the sequencing of nucleic acids (the so-called SBH method, sequencing by hybridisation).[132, 317–319] It was assumed that the use of arrays with a complete set of immobilised octanucleotides ($65536 = 4^8$) would make it possible to determine the unknown sequences of nucleic acids by hybridisation assays. However, it was found that serious problems are inherent in the SBH method and not much success in this area has been achieved yet. These studies have shown, however, that microchips can find wide application in the analysis of gene expression and mutations as well as in the diagnostics of genetic, infectious and cancer diseases.[320–334]

There are two main approaches to the preparation of DNA microchips, *viz.*, the synthesis of oligonucleotides directly on surfaces [4, 5, 8, 175, 215, 217, 218, 321, 333–341] and immobilisation of presynthesised oligonucleotides on slides of glass or other materials.[17, 19, 22, 30, 131, 133, 248, 322, 323, 330–332, 342–344]

The former approach has been realised in such methods as combinatorial photolithography on glass slides or other supports using photoreactive protective groups.[321, 337–341] Irradiation of slides through a cover mask results in the removal of photoreactive protective groups from the oligonucleotide chains at the predetermined sites so that the next monomeric unit is coupled to the deblocked oligonucleotide chain. By shifting the mask and by iterating the irradiation and coupling procedures, one can obtain ultimately surfaces containing a large number of oligonucleotides of predetermined sequences at specified points. Despite its obvious economic expediency, direct synthesis of oligonucleotides on surfaces suffers from one serious disadvantage, since it does not provide 100% yields in coupling steps, which can result in errors in further analysis.

The latter approach to the manufacture of DNA microchips entails the use of isolated and purified oligonucleotides.

Thorough pre-washing and etching prior to immobilisation is a very important stage. These are necessary for the removal of admixtures (grease, dirt, dust, *etc.*) and for the generation of surface hydroxy groups.[345, 346] The washing procedure makes use of organic solvents (most commonly, methanol) or potent oxidants (*e.g.*, hydrogen peroxide). The activation of the glass surface is achieved by inorganic acids or alkalis (HF, HCl, HNO_3, H_2SO_4 or NaOH). This is followed by the introduction of functional groups on the slide surface for subsequent reactions using silanes of the general formula $(R^1O)_3Si(CH_2)_nR$,[2] where R^1 = Me or Et and R^2 is a functional group (see compounds **1**–**4** in Section III.2). The reactions considered in the foregoing sections are used for covalent attachment of oligonucleotides onto the surface of glass slides. Here we shall cite a paper [344] which describes a novel approach. Oligonucleotides or cDNA with linkers containing 5′-terminal SH or $CH_2=CH$ groups were preincubated with the corresponding trimethoxysilanes, after which the conjugates obtained were immobilised on non-modified glasses.



R, oligonucleotide or cDNA.

The nature and the lengths of the linkers binding the oligonucleotide to the surface are important characteristics for effective hybridisation. Steric accessibility of oligonucleotides for complementary interaction can be achieved through the use of long-chain linkers. The efficiency of hybridisation increases severalfold through the use of spacers of appropriate lengths [8, 38, 320, 331–333] (see Section III.2). The amount of the immobilised oligonucleotide referred to the unit of surface area (the surface capacity) is yet another important characteristic of DNA chips; this should not exceed a certain optimum value in order to avoid steric hindrances during subsequent hybridisation. The capacity is not very high (0.01–1.00 pmol mm^{-2}) [8, 15–17, 19, 20, 242, 333] but sufficient for successful assays. The optimum capacity of 0.25–0.35 pmol mm^{-2} for the immobilisation of polynucleotides (150–350 b.p.) has been recommended.[17]

Obviously, the sensitivity of a hybridisation assay increases with an increase in capacity. This is achieved using several approaches, *viz.*, by attachment of oligonucleotides to branched linkers (dendrimers) [22] and by formation of acrylamide,[20, 29, 30, 45, 347–349] agarose [183] or gelatine [350] layers on glass surfaces, which ensures 'three-dimensional' attachment of oligonucleotides. In this case, the capacities of oligonucleotides referred to the unit of surface area is much higher than in two-dimensional immobilisation and can amount to 30 pmol mm^{-2},[45] while steric accessibility of oligomers for subsequent hybridisation is preserved.

In early studies, radiolabelled probes were used for the detection of signals in hybridisation assays. However, other labels incorporated into oligonucleotide probes, such as biotin, digoxigenin, chemiluminophores, fluorescent and enzymic labels, also gained wide acceptance.[302, 308, 351]

Physical methods of detection have come into use owing to the recent progress in DNA chip technology. The DNA sensors (biosensors), like other biosensors, provide rapid and direct detection both with labels and without them. The use of biosensors allows kinetic studies of hybridisation processes. The topology of immobilised oligonucleotides in films applied onto the surfaces of slides and fine details of molecular interactions at the film-solution interface are also investigated.[337–341, 352–356] Thus the effects of the shape, length and composition of the double helix formed on the efficiency of hybridisation have been studied.[4, 357, 358]

DNA sensors can utilise piezoelectric technologies for measuring the changes in the oscillation frequencies of crystals bearing immobilised oligonucleotides during the sorption of the complementary DNA chain,[359–362] surface plasmon resonance technology (in this case, the refractive index of the metal surface changes upon binding of the soluble ligand to the ligand immobilised on the metal film),[363–366] scanning microscopy [352, 367] and X-ray photoelectron spectroscopy (XPS).[353] Electrochemical detection makes use of electrodes bearing immobilised oligonucleotides which change their electrical characteristics in the course of hybridisation.[365, 368–370] Optical sensors able to detect changes

in the optical properties (*e.g.*, refractive index, interference, *etc.*) of films are also widely used for the detection of hybridisation of DNA chains on the surfaces containing immobilised oligonucleotides.[230, 371 – 373]

These methods ensure high sensitivity, *i.e.*, the detection of nano- and picogram quantities of compounds.

The design of DNA chips and DNA sensors is an explosively developing field of science and biotechnology. Advances in this area will be a breakthrough in the diagnostics of genetic, cancer and infectious diseases as well as in medicine and pharmacology.

4. Enzymic synthesis on NA templates

Immobilised synthetic oligonucleotides and NA as templates or primers for enzymic synthesis of oligo- and polynucleotides can repeatedly be used for the synthesis of complementary oligonucleotide chains, which simplifies the separation of the final product from the templates. The latter circumstance is especially important, since along with the template or the primer the reaction mixture contains the enzyme and a large excess of nucleoside triphosphates (NTP) used as substrates for NA biosynthesis.

DNA polymerases of bacteriophage T7,[34, 374] polymerase I of *E.coli*, the Klenow fragment [85, 257, 260, 375, 376] and Taq polymerase [377 – 380] are used in solid-phase enzymic synthesis of DNA or DNA fragments. cDNAs synthesised on oligo(dT)-bearing supports (*e.g.*, cellulose,[258, 259, 381] latex [382 – 384] or magnetic particles [374]) by transcription of the corresponding poly(A) – mRNAs in the presence of RNA-dependent DNA polymerase (reverse transcriptase) are most often used as templates. Some authors recommend to immobilise plasmids [34] or bacteriophages [192, 376] containing incorporated exogeneous DNAs (including cDNAs) [192] and utilise them as templates for subsequent enzymic synthesis on supports. Using primers complementary to the 3′-terminal region of the immobilised template, one can obtain full complementary DNA.[85, 192, 257, 380] Enzymic template synthesis permits one to introduce various functional groups and labels into DNAs and their fragments [257, 375] using primers, dNTP or terminating dideoxyribonucleoside triphosphates (ddNTP) carrying the corresponding functional groups. The addition of one or all the four ddNTP with different labels to the reaction mixture along with four dNTP makes it possible to obtain series of labelled products and to determine the oligonucleotide sequence of the immobilised DNA.[34, 378, 379]

The chemical synthesis of oligoribonucleotides and RNA is more complicated than that of oligodeoxyribonucleotides, hence, the interest in enzymic methods is obvious. Biosynthesis of RNA is carried out using DNA-dependent RNA polymerases, *e.g.*, RNA polymerases of T7 [129, 385, 386] and *E.coli*.[381, 387, 388] Immobilised oligodeoxyribonucleotides [129, 385, 386, 388] and cDNA are used as templates for oligoribonucleotide and mRNA syntheses, respectively.[381, 387] The amount of the final products can be increased by means of PCR technology, while modern magnetic particles (Dynabeads) ensure easier separation of the products synthesised.

Enzymic synthesis on immobilised NA templates is used for the replication, transcription and sequencing of nucleic acids as well as for the introduction of functional groups and labels and the construction of genomic and cDNA libraries.

VIII. Conclusion

The work considered in this review demonstrates the immense diversity of methods for immobilisation of NA on solid supports. Immobilisation of NA on activated supports are especially widely used. In this case, native NA is immobilised non-specifically through many points. However, this results in the preparation of affinity matrices with higher capacities. The attachment of nucleotides and oligonucleotides containing additional reactive groups occurs much more selectively, *i.e.*, without affecting the heterocyclic bases and with the preservation of their abilities for affinity interactions.

Physical immobilisation methods are irreproducible and non-specific. The advantage of chemical approaches is the possibility to use the immobilisation methods corresponding to a specified experimental task. The stability of affinity sorbents under various conditions and the procedure used for the removal of the ligand from the support can be determined in advance provided that the nature of the bond between the ligand and the support is known.

Reactions of ligands containing amino or thiol groups with the reactive groups of the support have become especially widespread among other methods of immobilisation of NA and their fragments. The attachment of oligonucleotides containing amino groups to supports containing aldehyde groups is a common procedure. The method utilising the $Ph_3P-Py_2S_2$ couple as an activating reagent has proved to be efficient in the binding of oligonucleotides with terminal phosphate groups to granulated polymers. In the case of planar supports, *e.g.*, films and glasses, it is more expedient to use methods implying the activation of the functional groups of the support or utilise bifunctional cross-linking reagents, *e.g.*, 1,4-phenylene diisocyanate. It should be noted, however, that no universal immobilisation procedure exists and both the support and the immobilisation procedure should be chosen for each particular case.

NA-containing carriers have found wide acceptance for both research and applied purposes. A number of reviews devoted to this topic has been published in recent years.[326, 328, 347, 389 – 391] The supports with immobilised oligonucleotides are used as sorbents in affinity chromatography in order to obtain highly purified individual NA or their fragments. Affinity chromatography seems to be the only efficient procedure for the separation of diastereomeric mixtures of non-ionic oligonucleotide analogues. Affinity oligonucleotide-containing matrices have proved to be highly efficient for the isolation of nucleotide-dependent enzymes. Such supports are also used in solid-phase enzymic synthesis of DNA, RNA and their fragments.

Special mention should be made of NA-containing carriers for hybridisation assays, which underlie the diagnostics of many diseases. It is used for detecting mutations, viral and bacterial NA, *etc.* This method is gaining increasing popularity for genetic expertise in medical and forensic practice.

The trend which includes the design and application of DNA microchips is developing explosively in recent years. These tools have proved to be useful in various fields of biology and medicine. Their unique properties are based on the fact that a small area of glass slides contains a large amount of immobilised oligonucleotides, which allows a great number of assays to be conducted simultaneously.

The use of immobilised oligonucleotides as biosensors adds novel unique capabilities to the arsenal of modern analytic techniques. They permit one to obtain information more rapidly, simply and less expensively than in conventional hybridisation techniques. The novel devices, *viz.*, DNA biosensors, hold tremendous potential in many areas, *e.g.*, clinical analyses for forensic expertise, monitoring of the environment, food quality, *etc.*

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