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POLYMERIC SUPPORTS BEARING ISONITRILE FUNCTIONAL GROUPS FOR COVALENT FIXATION OF BIOLOGICALLY ACTIVE MOLECULES (A REVIEW)

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SUMMARY

(1) Methods were developed for the introduction of isonitrile (isocyanide, $-N \equiv C$) functional groups on to the backbone of several types of natural and synthetic polymers —polysaccharides, polyamides, polyesters, polyvinylalcohols and polyacrylamides.

(2) Proteins and other high- and low-molecular-weight ligands were covalently linked to the various macromolecular isonitrile derivatives by four-component-condensation (4CC) reactions carried out in an aqueous medium of neutral pH values.

(3) The isonitrile groups on the support could be steered, by four-component coupling (involving amine, carboxyl, aldehyde and isonitrile), towards one type of ligand functional group (*e.g.* carboxyl or amine) by controlling the relative concentrations of the co-reagents.

(4) The isonitrile groups on the polymeric supports could be transformed into other types of functional groups by simple one-step reactions.

(5) The isonitrile groups on the polymeric matrix could also be utilized for the grafting of water-soluble macromolecules to obtain supports of modified surface properties.

INTRODUCTION

This paper summarizes methods for the introduction of isonitrile (isocyanide, –NC) functional groups on to the backbone of several types of natural and synthetic polymers: polysaccharides, polyamides, polyesters and acrylic polymers. Macromolecular supports containing isonitrile functional groups can be coupled to proteins and other high- or low-molecular-weight ligands by four-component condensation reactions[1–4] carried out in aqueous media at neutral pH and also in organic solvents; alternatively, they may serve as "parent polymers" for further chemical modification, as the –NC group on the polymeric reagent is easily transformed into other types of reactive groups according to the application envisaged[5–12].

The idea of immobilization of proteins by four-component condensation reactions originated in earlier work by Axen and Porath, who used low-molecular-weight water-soluble isonitriles to effect the coupling of enzymes to polymers containing carboxyl, amino or aldehyde functional groups[3,4].

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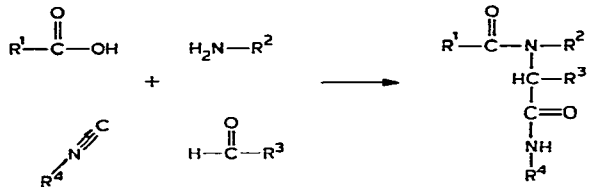
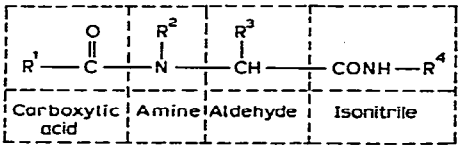
FOUR-COMPONENT CONDENSATION REACTIONS

Four-component condensations (4CC) involve amine, carboxyl, isonitrile and aldehyde (eqn. 1).



(1)

The carboxyl and amine moieties (R^1COOH and R^2NH_2) combine to form an N-substituted amide bond; the aldehyde and isonitrile components (R^3CHO and R^4NC) appear in the product as the side-chain attached to the amide nitrogen (see eqn. 2; R^4 in macromolecular isonitriles designates the polymeric support[1–13]).



(2)

The main advantage of four-component reactions stems from the fact that they can be steered in a given direction by controlling the relative concentrations of the co-reagents. This aspect is particularly useful when the product, an insoluble polymer, is easily removable from the reaction mixture[5–11].

Coupling of proteins and other ligands to polymers containing isonitrile functional groups

Proteins and other ligands can be bound covalently to polymers containing isonitrile functional groups by 4CC reactions carried out in an aqueous buffer at neutral pH in the presence of a water-soluble aldehyde, acetaldehyde[5–12]. The ligand may supply either the amino or the carboxyl component. The isocyanide group on the support is steered towards one type of ligand functional group by the addition of the missing fourth component, in excess, together with aldehyde, to the reaction medium (see eqns. 1 and 2). Thus, enzymes could be bound to polymers containing –NC groups, through protein amino groups by four-component reactions in the presence of acetaldehyde and excess of acetate (Table I); conversely, enzymes could be bound through their carboxyl groups in the presence of acetaldehyde and an amine such as tris(hydroxymethyl)aminomethane (Tris)[5–12] (see Tables II and III).

Four-component reactions based on multi-functional isonitrile support materials could also be used for the covalent fixation of unprotected peptides through either their $-NH_2$ or their –COOH termini by appropriate choice of coupling buffers[14] (see Table I). Similarly, oligo- and polysaccharides could be bound to polymers containing –NC functional groups, the reducing ends of the former serving as the aldehyde component in the 4CC reaction. Thus, mellibiose and dextran were coupled

TABLE I

BINDING OF GLYCYL-L-LEUCINEAMIDE AND TRYPSIN TO DIFFERENT POLYMERS CARRYING ISONITRILE FUNCTIONAL GROUPS

Support material	*Particle diameter (μm)*	*Bound peptide (μmole/g)*	*Bound protein*	
			Total (mg/g)	*Active (%)*
Nylon 6 powder	0.7 ± 0.3	45	48	63
Poly(ethylene terephthalate) powder	9 ± 3	48	67	30
Cellulose powder	56–63	31	12	55
Cross-linked dextran beads (Sephadex G-75)	40–120	30	9	100
Cross-linked dextran beads (Sephadex G-150)	40–120	33	13	70
Cross-linked agarose beads (Sepharose-CL, 4B)	40–190	63	91	30
Cross-linked polyacrylamide beads (Bio-Gel P-10)	75–150	40	20	70
Cross-linked polyacrylamide beads (Bio-Gel P-100)	75–150	60	70	45
Linear dextran (mol.wt. 250,000)	—	88	264	70
Poly(vinylalcohol) (mol.wt. 96,000)	—	98	197	80
Linear polyacrylamide (mol.wt. 200,000)	—	60	182	70

to polyisonitrile–nylon powder to give preparations containing 4–5 mg of carbohydrate per gram of support[14]. These aspects are of interest for the preparation of biospecific adsorbents.

With enzymes sensitive to aldehyde, protection from the deleterious effects of

TABLE II

BINDING OF ENZYMES TO NYLON DERIVATIVES CARRYING DIFFERENT FUNCTIONAL GROUPS

Nylon derivative	*Bound protein (mg/g)**				
	Trypsin	*Chymotrypsin*	*Subtilisin BPN'*	*Papain*	*Urease*
Isonitrile (4CC via $-NH_2$ Groups)	48 (63%)	45 (22%)	15 (35%)	69 (47%)	77 (1.5%)
Isonitrile (4CC via –COOH Groups)	44 (66%)	— —	7 (42%)	47 (30%)	110 (55%)
Dibromoisocyanide	21 (68%)	15 (66%)	9 (13%)	27 (15%)	62 (3%)
Acylhydrazide	16 (89%)	14 (74%)	9 (33%)	29 (42%)	70 (0%)
Aminoaryl	— —	— —	— —	86 (35%)	120 (92%)

* Numbers in parentheses are percentages of active protein.

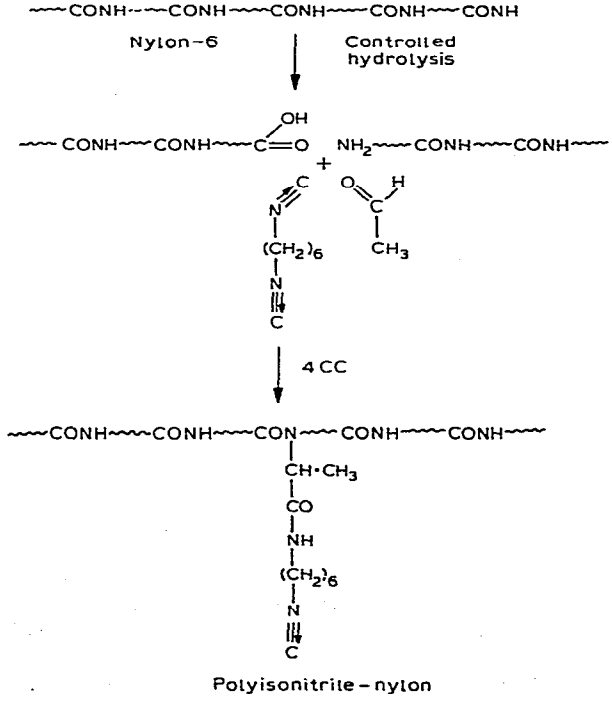
this co-reagent could be obtained by decreasing its concentration in the coupling mixture without significantly impairing the efficiency of binding. This is demonstrated in Table III for urease[11,12,14].

POLYMERIC SUPPORTS CONTAINING ISONITRILE FUNCTIONAL GROUPS

Isonitrile derivatives of synthetic polyamides

Nylon and related synthetic polyamides are in principle well suited as supports for the immobilization of enzymes and other biologically active molecules, owing to their high stability and mechanical strength[15]. The chemical inertness of the polyamide backbone, however, leaves only the terminal carboxyl and amino residues as possible functional groups for the covalent attachment of a ligand. Most of the published procedures for increasing the binding capacity of nylon (*e.g.*, by mild hydrolysis)[16–20] lead to some fragmentation of the polyamide backbone and may result in partial solubilization upon prolonged exposure to an aqueous medium; moreover, they result in residual charges on the polyamide backbone[18–20].

The method used by us for the introduction of isonitrile functional groups on the backbone of synthetic polyamides was based on (a) mild acid hydrolysis to generate $COOH \cdots NH_2$ pairs on the surface of a nylon structure and (b) resealing of the peptide bonds broken in the first step by a four-component condensation reaction (see eqn. 1) involving the neighbouring carboxyl and amino groups, acetaldehyde or isobutyral and a bifunctional isonitrile, 1,6-diisocyanohexane[5,6,9] (eqn. 3).



(3)

TABLE III

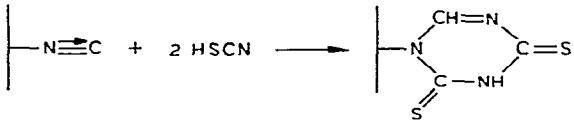
COUPLING OF UREASE TO ISONITRILE–CELLULOSE

Coupling mixture: urease (2 mg/ml) in appropriate buffer containing the specified concentration of acetaldehyde.

Coupling method	*Acetaldehyde concentration (M)*	*Bound protein*	
		Total (mg/g)	*Active (%)*
4CC via amino groups on protein*	0.9	25	6
	0.45	26	9
	0.18	26	10
	0.09	26	58
	0.045	26	65
4CC via carboxyl groups on protein**	0.9	18	–
	0.45	24	7
	0.18	36	51
	0.09	39	50
	0.045	41	52

* Buffer: 0.1 *M* sodium phosphate, 0.5 *M* in sodium acetate (pH 8).
** Buffer: 0.1 *M* Tris (pH 7).

This procedure leads to no fragmentation of molecules on the polymeric surface. The mechanical properties of derivatized nylons are thus unimpaired; morever, the chemically modified polyamide backbone carries no charged groups. The polyisonitrile–nylon preparations can be characterized by the direct titrimetric determination of the isonitrile groups present on the surface of the derivatized polyamide structure according to eqn. 4[7–10,21]:



(4)

or from the amount of dipeptide (*e.g.*, glycyl-L-leucineamide) bound by 4CC reaction under saturation conditions (see Table I).

Isonitrile derivatives of synthetic polyesters

Poly(ethylene terephthalate), a commercially available polyester, is a highly insoluble linear polymer that is used as a synthetic fibre and recently in prosthetic devices[22]. Again, no chemically reactive groups except for terminal carboxyl or hydroxyl groups are available for the covalent fixation of biologically active molecules. Isonitrile functional groups could be introduced on the surface of poly(ethylene terephthalate) powders of fibres by a procedure based on the Passerini reaction[1,23] in which carboxyl, aldehyde and isonitrile combine to form an ester substituted on the α-carbon of the alcohol moiety of the product (eqn. 5):

$$R^1COOH + R^2CHO + R^3NC \rightarrow R^1COOCH(CONHR^3)R^2 \quad (5)$$

The method consisted of (a) controlled alkaline hydrolysis to generate COOH ··· OH pairs on the surface of the polyester structure, (b) conversion of the hydroxyl groups into aldehydes by controlled oxidation and (c) resealing of the carboxyl–aldehyde pairs by a Passerini-type reaction using 1,6-diisocyanohexane [R^3 in eqn. 5 = $(CH_2)_6NC$][10].

Isonitrile derivatives of polysaccharides

Polysaccharides such as cellulose, cross-linked dextran and agarose beads are among the most widely used supports for enzyme immobilization and affinity chromatography[24–26]. The procedure most commonly used in the laboratory for the preparation of insoluble ligand–polysaccharide conjugates is the cyanogen bromide activation method[27–31]. Despite its numerous advantages, this method poses some problems, owing to the leakage of covelently bound molecules and particularly low-molecular-weight ligands, observed when polysaccharide conjugates are exposed to nucleophiles or alkaline pH[24–34]. This phenomenon has been attributed to the instability of the N-substituted isourea structures formed in the coupling reactions between ligand amino groups and the cyanogen bromide-activated support[24–32].

The procedure developed in our laboratory for the preparation of isonitrile derivatives of polysaccharides and other hydroxylic polymers involved ionization of hydroxyl groups on the polymer by a strong base in a non-aqueous solvent [sodium *tert.*-butoxide in dimethyl sulphoxide (DMSO)], followed by a displacement reaction via nucleophilic attack of the alkoxide ions on a low-molecular-weight isonitrile containing a good leaving group in the ω-position: 1-tosyloxy-3-isocyanopropane (p-$CH_3C_6H_4SO_2O(CH_2)_3NC$)[11]. The reaction sequence leading to polyfunctional 1-oxa-3-isocyanopropane ($-OCH_2CH_2CH_2NC$) derivatives of polysaccharides is summarized in eqn. 6:

(6)

Tos = p-$CH_3C_6H_4SO_2$–

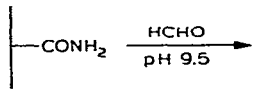
By this method, the side-chains containing the –NC functional groups are attached to the polymer backbone by stable ether bonds[11].

Isonitrile derivatives of polyacrylamides

Acrylic polymers and specifically cross-linked polyacrylamide polymers and copolymers are of interest as supports for the immobilization of enzymes and as bioaffinity matrices, owing to their chemical and mechanical stability and particularly their inertness to microbial degradation[35].

Isonitrile derivatives of cross-linked polyacrylamide beads and of linear polyacrylamide were prepared by a procedure consisting of partial hydroxymethylation of amide groups on the polymer by treatment with aqueous formaldehyde at alkaline pH (eqn. 7), ionization of the hydroxyl groups thus generated and then treatment with 1-tosyloxy-3-isocyanopropane in dimethyl sulphoxide (eqn. 8) in a reaction anal-

ogous to that employed for polysaccharides and other polyalcohols (compare with eqn. 6)[36].



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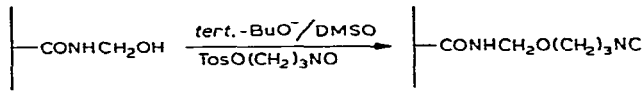
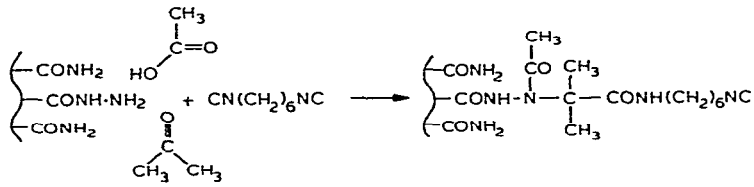


(8)

Isonitrile derivatives of insoluble polyacrylamide preparations and particularly acrylamide grafted on to rigid supports (as for example in eqn. 14) could be prepared by an alternative method; a fraction of the amide groups on the support was converted into acylhydrazide by controlled hydrazinolysis[37,38] and the –NC group introduced by a four-component reaction in which acylhydrazide groups on the polymer served as the amine component; acetic acid and 1,6-diisocyanohexane supplied the carboxyl and isonitrile functions and the solvent, acetone, supplied a large excess of the carbonyl component (eqn. 9).



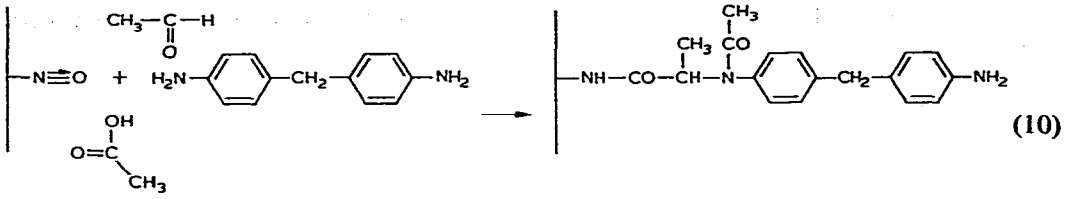
(9)

CHEMICAL MODIFICATION OF POLYMERS CONTAINING ISONITRILE FUNCTIONAL GROUPS

The coupling of enzymes and other proteins to polymeric supports containing isonitrile functional groups by four-component reactions in an aqueous buffer suffers from one disadvantage: the method can be used only when the biological activity of a protein is not impaired in the presence of aldehyde. The isonitrile functional group, however, can easily be transformed into other types of chemically reactive groups. Two alternative routes for the transformation of –NC groups were developed. These methods were particularly useful in cases where the presence of aldehyde in the coupling mixture had to be avoided. Most of the work reported below was carried out with polyamides of the nylon 6 and 66 type.

Modification of isonitrile groups by four-component reactions

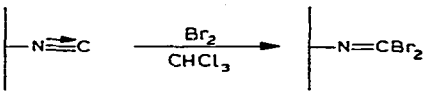
The isonitrile groups on a polymer can be converted into other chemically reactive groups by 4CC reactions in which one of the components is a bifunctional reagent. This approach is demonstrated by the conversion of polyisonitrile–nylon into a diazotizable polyaminoaryl derivative by a four-component condensation with a bifunctional aromatic amine, *p,p'*-diaminodiphenylmethane, in the presence of an aldehyde and a carboxylate[6,9,10] (eqn. 10). Coupling of enzymes to this nylon derivative is effected by diazotization (Table II).



(10)

Modification of isonitrile groups through the formation of dihaloisocyanide derivatives

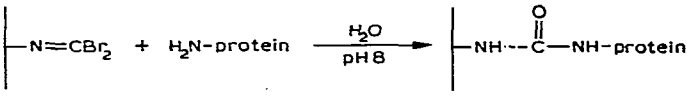
Isonitriles can easily be converted into the strongly electrophilic dibromoisocyanides by treatment with bromine in an inert solvent (eqn. 11).



(11)

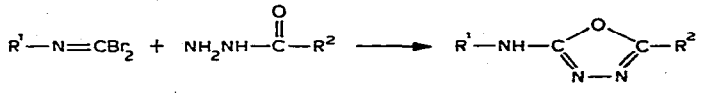
Dibromoisocyanide polymers, in effect the polymeric structural analogues of phosgene, can be used for the coupling of proteins and other ligands under mild conditions (Table II)[7–9,12].

With proteins, coupling to dibromoisocyanide polymers was shown to occur mainly via lysine ε-amino groups (eqn. 12), imidazole groups of histidine and tyrosine hydroxyls as well as via sulphydryl groups[7]. Considerable selectivity towards the various amino acid residues could be attained by monitoring the pH of the reaction[7].



(12)

Alternatively, the reaction of isocyanide dihalides with acylhydrazides[1,39] to form five-membered heterocycles of the 1,3,4,-oxadiazole type (eqn. 13) can be utilized for additional chemical modifications. Thus, acylhydrazide derivatives of nylon were prepared by reacting the dibromoisocyanide derivative of the polymer with the hydrazide of a dicarboxylic acid, *e.g.*, adipic dihydrazide [R^2 in eqn. 13 = $-(CH_2)_4CONHNH_2$][7–9,12] (see Table II).



(13)

GRAFTING OF WATER-SOLUBLE MACROMOLECULES ON TO PRE-FORMED POLYMERIC STRUCTURES

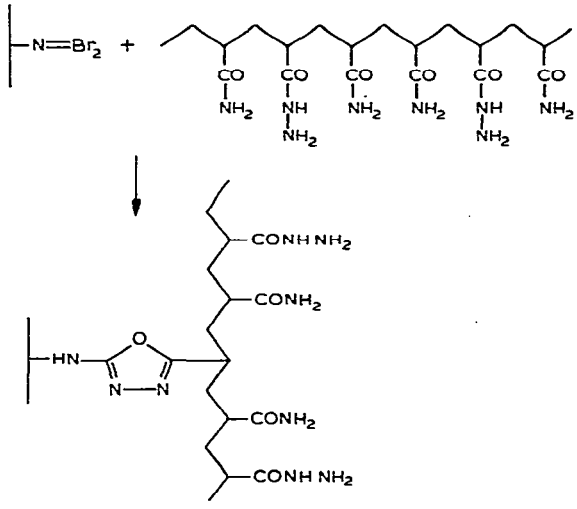
The versatility inherent in the chemistry of the isonitrile functional group can be utilized for the modification of the surface properties of support materials through the grafting of different types of linear macromolecules on to pre-formed polymeric structures. Two approaches, based on the reactions used heretofore, were investigated[36], as described below.

Grafting of water-soluble polymers containing isonitrile functional groups on to rigid supports

Isonitrile derivatives of linear, water-soluble polysaccharides, poly(vinyl alcohol) and linear polyacrylamide could be grafted directly on the surface of nylon structures by a 4CC reaction analogous to the procedure outlined in eqn. 3, *i.e.*, controlled acid hydrolysis to generate COOH $\cdots$ NH_2 pairs on the nylon surface, followed by a four-component reaction in the presence of the water-soluble polymeric isonitrile and an aldehyde, whereby the carboxyl–amine pairs are resealed by 4CC with some of the –NC groups of the macromolecular isonitrile (see Table IV).

Grafting of water-soluble macromolecules on to rigid supports containing isonitrile groups

Polyacrylamide. Polyacrylamide–nylon grafts were prepared essentially as outlined in eqn. 13. Linear polyacrylamide in which a fraction of the amide groups were converted into acylhydrazide by controlled hydrazinolysis[37,38] was reacted with dibromoisocyanide–nylon to yield a graft where the two polymeric moieties were bridged via 1,3,4-oxadiazole rings (eqn. 14)[8,9].



(14)

The unreacted acylhydrazide groups could be utilized to bind ligands by the azide method[8,26], they could be activated with glutaraldehyde, or they could be converted into isonitrile groups by the procedure outlined in eqn. 9 (see Table IV)[26,36].

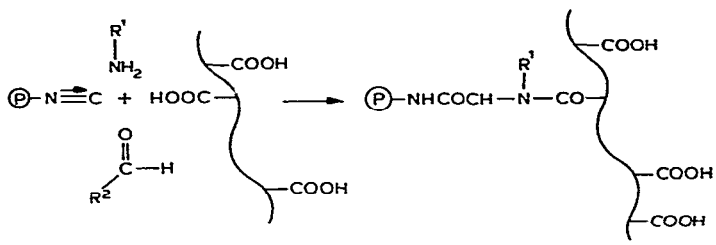
Polysaccharides. Linear dextran could be grafted on polymers containing –NC functional groups by four-component reaction (eqns. 1 and 2) in an aqueous medium containing acetate and aminoethanol or Tris as amine donor, the carbonyl component being supplied by the reducing ends of the polysaccharide (see also the section *Coupling of proteins and other ligands to polymers containing isonitrile functional groups*)[36]. Non-specific adsorption of proteins such as haemoglobin or bovine serum albumin on dextran-grafted polyamide and polyester particles or fabric was considerably lower than that observed with the unmodified polymers.

TABLE IV

BINDING OF GLYCYL-L-LEUCINEAMIDE AND TRYPSIN TO DIFFERENT WATER-SOLUBLE POLYMER–NYLON GRAFTS

Linear macromolecule	*Isonitrile content (μmole/g)*	*Bound peptide (μmole/g)*	*Bound protein*	
			Total (mg/g)	*Active (%)*
Polyacrylamide (acylhydrazide derivative) modified according to eqn. 10	17	17	27	70
Polyacrylamide (isonitrile derivative)	15	14	23	48
Dextran (isonitrile derivative)	14	14	20	60
Polyacrylic acid (isonitrile derivative)	–	28	32	70
Polyvinylamine (isonitrile derivative)	–	22	30	65

Polycarboxylates and polyamines. Polyacrylic acid could be grafted on to isonitrile–nylon derivatives by a four-component reaction in which polyacrylic acid supplied the carboxyl component and the reaction medium contained aldehyde and an amine (Tris or ethanolamine)[36].



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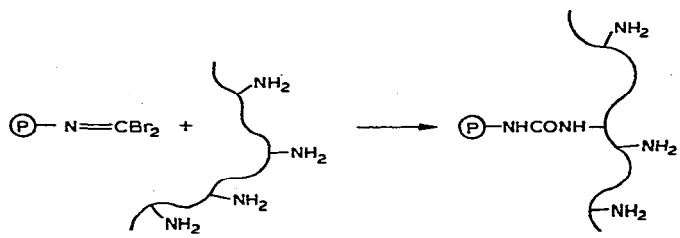
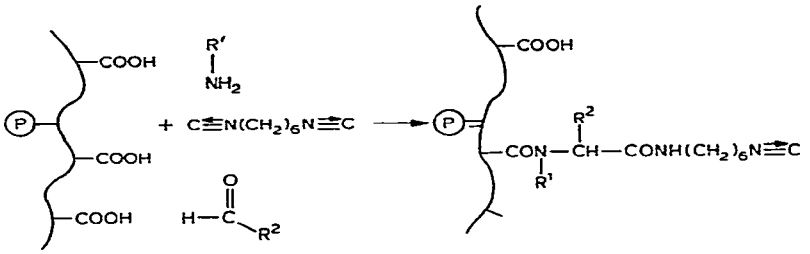
Polyvinylamine–nylon grafts could be prepared by reaction of dibromoisocyanide–nylon with polyvinylamine (compare with eqn. 18)[36].



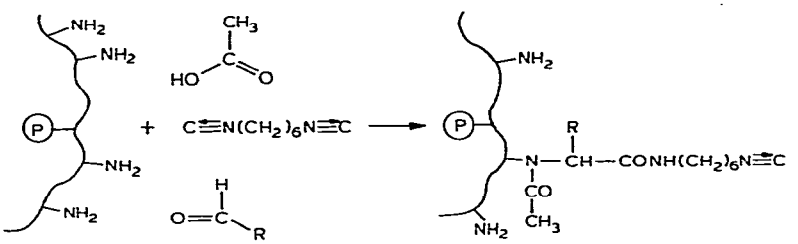
(16)

Isonitrile functional groups could be reintroduced on the polyelectrolyte–nylon grafts by additional 4CC reactions in the presence of 1,6-diisocyanohexane, aldehyde and amine or carboxyl for the polyacrylic acid and polyvinylamine grafts, respectively (eqns. 17 and 18)[36].



(17)

The functionalized graft polymers served as supports for the attachment of enzymes and peptides (see Table IV).



(18)

CONCLUSION

Polymers containing isonitrile functional groups offer several advantages as supports for the covalent fixation of high and low-molecular-weight ligands: (i) the side-chains containing the isonitrile function are attached to the polymeric backbone by stable bonds; (ii) the –NC groups generate peptide bonds in four-component reactions involving the ligand moiety; (iii) ligand–polymer conjugates carry no residual charged groups deriving from either modification or coupling reaction.

The use of four-component reactions in conjunction with polymers carrying pendant isonitrile groups allows covalent fixation of ligands through both –COOH and $-NH_2$ functional groups[6–12,14]. With enzymes sensitive to aldehyde, protection against the harmful effects of this reagent can be obtained by decreasing its concentration, without impairing the efficiency of binding. The –NC moiety on a polymeric matrix can also be transformed into many other types of reactive groups by four-component and other simple one-step reactions[5–12].

The methods described here can be easily adapted to a wide range of preformed polymeric structures, *viz.*, powders, pellets, beads, fibres, sheets, etc. The application of immobilized proteins and other ligands in a variety of configurations without significantly changing immobilization parameters (*i.e.*, nature of the support material and the method of coupling) is thus possible. The virtues of this approach could be demonstrated in studies aimed at the development of enzyme reactors and biospecific solid-phase assay procedures. Urease was bound to spun-bonded nylon fabric filters. Because of the low hydrodynamic resistance of the nylon fabric, it was possible to construct continuous-flow multi-layer enzyme reactors that could be operated at levels of up to 95% urea hydrolysis over a wide range of substrate concentrations (5–250 m*M*), flow-rates (5–100 ml/min) and column heights (20–200 urease nylon filters)[40].

In a similar approach, pectinesterase and polygalacturonase were immobilized on nylon net to give preparations of high enzymic activity and excellent stability[41]. Continuous experiments with the immobilized pectolytic enzymes acting on their natural substrate strongly suggested that such systems could be used for the clarification of fruit juices on an industrial scale, particularly in view of the fact that no inhibitory effects arising from the presence of polyphenols in the medium were observed.

The quantitative determination of biotin by an assay procedure based on avidin covalently attached to filter-paper discs has recently been reported[42]. Avidin–cellulose discs were incubated in biotin solutions of unknown concentration, washed to remove unbound material and then saturated with [^{14}C]biotin. Based on the known capacity of the discs for [^{14}C]biotin, the amount of unlabelled biotin extracted from solution by the avidin–filter paper discs could easily be estimated. The 4CC approach used to immobilize the protein (through its carboxyl groups) satisfied the stringent requirements for reproducibility and long-term stability.

The potential of multi-functional isonitrile reagents has yet to be explored. By appropriate choice of polymers and modification procedures, derivatized polymers of different chemical and mechanical properties can be synthesized as suggested by the work of Skorna and co-workers[43,44].

Further, as macromolecules containing $-N\equiv C$ groups can be viewed as the polymeric analogues of carbon monoxide, such polymers and specifically polymers containing rigid bidentate isonitriles could serve as templates for polymeric coordination compounds of transition metals.

Multi-functional isonitrile reagents can thus offer great flexibility and versatility in designing convenient procedures for the preparation of biospecific adsorbents and new polymeric reagents as well as novel synthetic approaches via enzymic and particularly organometallic catalysis. First steps towards these goals have been reported recently[45–48].

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