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MODIFICATION REACTIONS APPLICABLE TO POLYMERIC MONOLITHIC COLUMNS. A REVIEW

Sinéad CURRIVAN and Pavel JANDERA[1]
Department of Analytical Chemistry,
The University of Pardubice, CZ–532 10 Pardubice

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Organic monolithic columns can be prepared from co-polymerizations of functional monomer and a cross-linker, resulting in a desired surface functionality. They can also be prepared to form a generic polymer monolith, in which the functionality can be altered by using a number of post-polymerization modifications. The reactions used to convert surface functionality include reaction chemistry, thermal or photo-initiated polymerization reactions, or the addition of nano-architectures, such as nano-particles or nano-tubes. This review article aims to highlight the available options in the modification of polymeric monolithic columns prepared by thermal or UV initiated polymerizations.

Contents

[1] To whom correspondence should be addressed.

Introduction

Organic polymer monolithic columns were first developed by Hjertén in the late 1980's [1], and were advanced further throughout the early 1990's [2-5]. Porous polymer rods have come to include sophisticated designs for columns and microfluidic devices, which contain not only multifunctional surface chemistries [6-9], but also gradients of surface functionality [10-13], separate to the column morphology. Since the introduction of polymeric monoliths, many reviews regarding monolith fabrication have been written with focused reviews on preparation [14-18], modification reactions [19-22], with consideration towards particular applications such as ion exchange [19,20], solid phase extraction [22,23], biomolecule immobilization [22,24-26], and the role of nano-particles in separation science [27,28] and biomolecule immobilization [29]. This review is aimed at highlighting varieties of post-polymerization modifications available for

polymeric monolithic columns, prepared by free radical polymerization *via* thermal or UV initiated methods.

The use of post-polymerization modification has, in recent years, become a commonplace technique in the preparation of monolithic separation media. The goal of post-polymerization modifications is to separate the control of morphology from the selectivity, modification, and tailoring of the surface functionality [30]. Modifications of this type can result in monoliths with readily optimized morphologies, as well as increased surface density of desired functional groups [7,30] leading to increased efficiency.

Co-polymerization of a desired monomer with a cross-linker, and/or a second functional monomer is the most technically straight forward method to incorporate the desired surface functionality [4,5,11,30,31]. Monolithic columns prepared directly from a monomer of desired functionality, and cross-linker have also been reported [32-37]. Co-polymerization is a well-established method for the preparation of monolithic columns expressing the preferred functionality [30]; however, as polymeric monoliths can express a low surface availability of functional groups, as they contain a high population of large through-pores, and as such they have limited surface areas [2]. The available surface functionality can be limited, as monomers with specific functionality may not always be commercially available, and synthesis may not be a viable option [15]. To generate these preferred surface chemistries, columns have been subsequently modified following preparation of a "generic" base monolith [38,39,40]. A number of techniques such as reactions with pendant surface groups, grafted chain growth, hypercross-linking, or the introduction of nano-architectures to the formed monolith, can be used to tailor the desired surface functionality. Post-polymerization modification can be defined as those reactions confined to or emanating from the surface of the monolithic column, using (a) reaction chemistry (e.g., glycidyl methacrylate (GMA), *N*-acryloxysuccinimide (NAS), vinyl azlactone (VAL)) from a functional group incorporated into the monolith structure, (b) grafting of additional polymer layers by means of thermal or photo-initiated polymerizations, or (c) the addition of nano-architectures to aid in selectivity, or to enhance surface area.

2 Reactive monolithic columns

A reactive monomer such as GMA [2,3], VAL [5,6,11,41], or NAS [42-45], can be incorporated into the monolithic matrix, which can be further modified to express a preferred surface functionality. GMA has been used as a co-monomer in monolithic column preparations, since the introduction of rigid monolithic columns [2], with varying modification reactions performed *in situ*. The reactive GMA group is generally used to convert monolithic columns to ion exchange

selectivity [2,30,46-53], affinity selectivity [54-58], or in the immobilization of zwitterionic molecules, for protein separations in capillary electrochromatography (CEC) [59-61]. Such columns can find applications in microLC, or CEC.

Using the reactive monomer in a modification reaction is known for its relative ease in preparation; however, Svec reported that it generally yields a low surface coverage [15], as the reliant chemical group (e.g., oxirane) is not only expressed sporadically on the globular surface of the monolith, but inaccessible, within the bulk of the globule. This was further emphasized by Rohr *et al*. [39] who noted that the use of potential reactive sites on the surface of the monolith for further modification was first dependent on the primary coverage of such sites. With a monolith prepared *via* co-polymerization, in order to increase the surface density, the alteration of the monolith components (such as monomer concentration and porogen ratios) would be required, which thus results in significant changes in the monolith morphology [39]. Reaction chemistry results in the conversion of only the available reactive sites, generated by the initial polymerization procedure, compared to a secondary surface polymerization, e.g., grafting, wherein a network of new functional groups can be generated.

2.1 Ion exchange chromatography

The versatility of the pendent epoxide groups of GMA incorporated monolithic columns leads to the vast range of applications found in the literature (Table I). The introduction of this technology by Svec *et al*. [2] has led to the preparation of such monolithic columns for the ion exchange (IEX) separation of oligonucleotides [51,53], small inorganic cations [47] and anions [30]. The first reported monolithic column prepared by Svec *et al.* [2] in 1992 was prepared from GMA and ethyleneglycol dimethacrylate (EDMA) monomers. The resulting column had a modest surface area of 10 $m^2 g^{-1}$. The pendant epoxide rings were aminated to prepare an anion exchange column, and a range of proteins were separated upon the column. In a subsequent report, Svec *et al.* [3] prepared poly(GMA-*co*-EDMA) monolithic columns which were again aminated. The monolith was used in the separation of three proteins; lysozyme, soyabean trypsin inhibitor, and conalbumin, and demonstrated baseline separation within 18 min (protein concentration at 2.5 mg each). Similarly, Sýkora *et al.* [46] developed poly(GMA-*co*-EDMA) monolithic columns, which were aminated with diethylamine. The conversion of epoxide groups was investigated across three columns (columns I-III), using changes in reaction conditions such as temperature and time. The group clearly demonstrated the relationship between increased time and temperature of reaction, with the resulting higher surface coverage of desired functionality. A disadvantage of the technique was noted; the loss of a large proportion of epoxide groups which were held within the monolith bulk structure

(by virtue of co-polymerization). The change in the monolith's surface concentration of amino groups was reflected in the retentive ability of each column type, with column I (3 h, 55 °C) having a greater retention than both column II (1 h, 55 °C), and column III (5.5 h, 20 °C).

Table I Reactions used in formation of ion exchange stationary phases for polymeric monolithic columns. RT refers to room temperature

Monolith	Reaction	Temperature °C	Time h	Application	Ref.
Poly(GMA-*co*-EDMA)	Amination with diethylamine.	70	3	Ion exchange of proteins.	[2]
Poly(GMA-*co*-EDMA)	Amination with diethylamine.	70	8	Ion exchange of proteins.	[3]
Poly(STY-*co*-DVB)	Sulfonation with chlorosulfonic acid in dry dichloromethane.	RT	20	Separation of inorganic anions.	[30]
Poly(GMA-*co*-EDMA)	Sulfonation with 4-hydroxybenezenesulfonic acid and triethylamine.	60	20	Separation of inorganic anions.	[30]
Poly(GMA-*co*-EDMA)	Sulfonation with thiobenzoic acid and triethylamine, followed by oxidation using a solution of tert-butylhydroperoxide.	60	20	Separation of inorganic anions.	[30]
Poly(GMA-*co*-EDMA)	Sulfonation with sodium sulfite, containing tetra-*n*-butylammonium hydroxide.	75	20	Separation of inorganic anions.	[30]
Poly(GMA-*co*-EDMA)	Amination with diethylamine using varying time and temperature.	55 (I and II) 20 (III)	3, 1, 5.5	Separation of oligodeoxyadenylic acids.	[46]
Poly(GMA-*co*-EDMA)	Sulfonation with Na_2SO_3.	75	N/A	Inorganic cations and cations in human saliva.	[47]
Poly(GMA-*co*-DVB)	Amination with Na_2CO_3, diethylamine and NaCl.	60	8	Separation of oligodeoxyadenylic acids.	[51]
Poly(GMA-*co*-DVB)	Amination with Na_2CO_3, diethylamine and NaCl. Column was further alkylated to express a quaternary ammonium.	60	8	Nucleotide and oligonucleotide separations	[53]

Bisjak *et al.* [51] developed an aminated poly(GMA-*co*-divinyl benzene) (poly(GMA-*co*-DVB)) monolithic column for anion exchange chromatography. The column was applied to the separation of phosphorylated oligothymidylic acids, eluting in less than 10 min. Later, a poly(GMA-*co*-DVB) monolith was prepared and subsequently aminated [53]. The resulting tertiary amine was further alkylated to produce an anion exchange functionality using a solution of diethyl sulphate in nitromethane. The column was applied to the separation of oligothymidylic acids, as in their previous work. In addition, mono-, di-, tri-phosphates, and nucleotides were separated. The resulting column efficiencies were between 25,000 and 30,000 N m^{-1} for adenosine triphosphate (ATP).

Ueki *et al*. [47] prepared a poly(GMA-*co*-EDMA) monolithic column, similar to the work by Sýkora *et al*. [46], and experimented with the conditions used for column modification, in this case sulfonation for cation exchange chromatography. Surface modification was achieved by flushing a solution of Na_2SO_3 through the column. The resulting ion-exchange capacity could be controlled by varying reaction conditions such as concentration of Na_2SO_3 modifier, and time for reaction. Columns of varying capacity were prepared, and ranging from 5.5 μequiv ml^{-1} to 151 μequiv ml^{-1}, with only 13 % of the GMA monolith content being converted. This may be due to the inherent fault in the method of co-polymerization, wherein the majority of functional groups are in the bulk of the monolithic material, and not available on the surface of the monolith [46]. The column was successfully applied to the detection of cations in human saliva, with detection of sodium, potassium, calcium, and magnesium cations, providing an efficiency of 20,000 N m^{-1}.

3 Monolithic Columns Immobilized with Biomolecules

Reactive co-monomers can be used in a polymerization reaction for the direct modification of the resulting surface reactive sites. The majority of epoxide modified stationary phases, account for stationary phase development in affinity applications, wherein a biomolecule of interest is immobilized to the inner monolith surface [24]. A number of possible reactions were described in detail in an excellent review published by Mallik *et al.* [24]. A summary of reactions can be seen in Table II.

3.1 Monoliths with Immobilized Enzymes

In 1996, Petro *et al.* [62] immobilized trypsin to the inner surface of a poly(GMA-*co*-EDMA) monolithic column, for applications in protein digestion and affinity chromatographic applications in the separation of trypsin inhibitor. Free trypsin,

trypsin modified beads, and a trypsin modified monolith, were used in the digestion of cytochrome C. Using reversed-phase (RP) chromatography, cytochrome C and cytochrome C digests were separated and compared. The trypsin modified columns were used successfully in protein digestion, with immobilized trypsin more stable than its dissolved counterpart. The trypsin immobilized monolith demonstrated superior proteolytic activity, due to enhanced mass transfer [62].

In addition to the immobilization of biomolecules *via* epoxide rings, other reactive monomers can be incorporated into the monolith matrix, including vinyl azlactone (VAL). Xie *et al.* [5] produced a monolith using co-polymerized VAL, with acrylamide and EDMA monomers. Following the formation of the monolith, trypsin was immobilized to the pendant reactive VAL sites, producing an enzymatic micro-reactor. By immobilizing trypsin to a monolith, the group evaluated the enzyme activity following a change in reaction conditions such as flow velocity, temperature, and pH. The greatest activity was investigated and found to be at a higher temperature than previously reported for free trypsin in solution (75 °C vs 60 °C, respectively), whilst varying the pH had little or no effect on the immobilized enzyme, relative to its counterpart in solution. By immobilizing such enzymes to monolithic supports, an increase in stability of the enzymatic reaction can be obtained.

Křenková *et al.* [63] also immobilized trypsin using two different processes on GMA stationary phases. In the first approach, the enzyme was immobilized directly to the monolith, whilst in the second approach, an aldehyde was generated on the surface for immobilization. The digestion procedure was found to occur relatively faster in-column than solution based digestion (30 s versus 3 h, respectively), similar to the report by Petro *et al*. [62]. Peterson *et al*. [6] developed a microfluidic reactor, with VAL co-polymerized with acrylamide or hydroxyethyl methacrylate (HEMA), with EDMA as a cross-linker. Trypsin was immobilized to the monolith surface *via* covalent linkages from the VAL functionality, *via* nucleophilic attack. The trypsin micro-reactor was used in the digestion of equine myoglobin, and was subject to MALDI-TOF-MS identification. The group investigated the reactor's capability, activity, and methods to increase activity. By increasing the percent concentration of VAL in the monolith precursor solution, an increase in activity was observed, due to an increased quantity of immobilized trypsin.

Palm *et al*. [43] prepared a monolith of poly(acrylamide-*co*-methylene-bisacrylamide-*co*-polyethyleneglycol-*co*-NAS), in which the reactive monomer NAS was used for the subsequent immobilization of PNGase F enzyme, suitable for deglycosylation of *N*-linked oligosaccharides. In this modification, the enzyme was also added to the monolith precursor solution, reacting with NAS whilst in solution. The group used the monolith successfully in the deglycosylation of glycoproteins such as ribonuclease B, asialofetuin, and ovalbumin, with MALDI-MS detection.

3.2 Monoliths for Lectin Affinity Separations

Columns incorporating lectins or glycans are important as many glycoproteins have been detected as biomarkers in diseases, and as such, the extraction of such molecules for analysis is vital. Pan *et al*. [55] immobilized a lectin, protein A, to poly(GMA-*co*-EDMA) monoliths. The latent epoxide rings were firstly hydrolysed, and then oxidized to form aldehyde groups on the monolith's surface, to which the lectin was bound. These columns were used in the specific retention of IgG.

Bedair and co-workers [56] exploited the highly efficient affinity of lectins to selectively trap and elute lectins from a mannose modified monolith. Poly(GMA-*co*-EDMA) and poly(GMA-*co*-methacryloyloxyethyl trimethylammonium chloride-*co*-EDMA) (poly(GMA-*co*-META-*co*-EDMA)) monolithic columns were prepared which were subsequently modified with polyethylene glycol (PEG) 200 (for neutral monoliths), or triethylenetetramine (in META containing columns). Using the selective affinity (in either CEC or LC), a number of chromatograms were collected, demonstrating the high specificity of the column towards mannose binding proteins. The extraction of mannose binding protein from rabbit serum was also demonstrated, with very little sample clean-up. The eluted fraction was collected and subjected to SDS-PAGE for further confirmation. In 2005, Bedair *et al*. [57] again reported the use of lectins in affinity chromatography. Two lectins, concanavalin A (Con A), and wheat germ agglutinin (WGA), were immobilized to poly(GMA-*co*-EDMA) and poly(GMA-*co*-META-*co*-EDMA) monoliths. The selectivity of the lectin immobilized supports was demonstrated by the extraction of glycoproteins and glycans for pre-concentration, from dilute solution in LC experiments. In addition, Con A-RPLC 2D separations of glycoproteins and proteins were performed. The chromatograms demonstrated the separation ability of these affinity columns, with glycoproteins trapped on the lectin affinity column, and three non-retained proteins were subsequently separated on the second dimension, C_{18} RP column.

3.3 Monoliths for Other Affinity Chromatographic Separations

Avidin was immobilized to either poly(GMA-*co*-acrylamide-*co*-EDMA) or poly(GMA-*co*-EDMA) monolithic columns, *via* epoxide chemistry, using glutaraldehyde as a spacer arm for avidin immobilization [64]. Columns prepared with acrylamide demonstrated higher biotin binding capacity, as glutaraldehyde may have reacted with pendant amide groups from acrylamide. The monolithic columns were used in the purification of biotinylated cytochrome C, where monoliths prepared with acrylamide and GMA showed a lower recovery than those prepared with GMA. However, the group reported that their media performed better than those commercially available.

Table II Reactions of reactive monoliths in formation of biomolecule expressing stationary phases. RT refers to room temperature

Monolith	Reaction	Time h	Application	Ref.
Poly(acrylamide-*co*-VAL-*co*-EDMA)	Immobilization of trypsin.	1	Activity of immobilized trypsin determined using L-benzoyl arginine ethyl ester and casein.	[5]
Poly(acrylamide-*co*-VAL-co-EDMA)	Immobilization of trypsin.	1-8	Tryptic digest of equine myoglobin for MALDI-TOF detection.	[6]
Poly(VAL-*co*-HEMA-*co*-EDMA)	Immobilization of anti-testosterone polyclonal antibody.	1	On-line immuno-extraction.	[41]
Poly(acrylamide-*co*-methylenebisacrylamide-*co*-NAS)	PNGase F modification of surface poly(NAS).	0.5	Deglycosylation of proteins prior to MALDI-TOF detection.	[43]
Poly(GMA-*co*-TRIM) and Poly(GMA-*co*-EDMA)	Immobilization of Protein A.	16	Isolation of hIgG from human serum.	[55]
Poly(GMA-*co*-EDMA) and Poly(GMA-*co*-EDMA-*co*-META)	Immobilization of mannan.	16	Trap and release of concanavalin A and *lens culinaris* agglutinin.	[56]
Poly(GMA-*co*-EDMA)	Immobilization of lectins (concanavalin A, wheat germ agglutinin).	16	Trap and release of glycosylated proteins.	[57]
Poly(GMA-*co*-EDMA)	Immobilization of trypsin.	4	Digestion of cytochrome C.	[62]
Poly(GMA-*co*-EDMA)	Immobilization of TPCK-trypsin	3, 4	Digestion of cytochrome C.	[63]
Poly(GMA-*co*-AMPS-*co*-EDMA)	Immobilization of anti-ochratoxin A and anti-alflatoxin antibodies	17	Immuno-preconcentration of ochratoxin A.	[65]

Not only can reactive monoliths be used in IEX or for the production of micro-reactors, but in the preparation of numerous other applications. For example, iminodiacetic acid (IDA) was immobilized to poly(GMA-*co*-EDMA) monolithic columns by Lou and co-workers for immobilized metal affinity chromatography (IMAC) applications [54]. The column was used in IMAC separations, with copper complexed IDA and nickel complexed IDA, with both chromatograms showing differing selectivities. Fractions of human serum albumin (HSA) were then purified and detected using MALDI-TOF-MS, which determined a high purity of HSA.

3.4 Monoliths with Immobilized Antibodies

Antibodies can also be immobilized using pendent epoxide rings. Faure *et al.* [65] used poly(GMA-*co*-AMPS-*co*-EDMA) monoliths to immobilize anti-alflatoxin antibodies, and anti-ochratoxin A antibodies, *via* direct interaction with the pendent epoxide groups. Following affinity applications, the immunosorbent was found to be selective, as only the column with immobilized antibodies showed retention, versus two other columns: one column of non-specific antibodies, and one un-modified bare poly(GMA-*co*-AMPS-*co*-EDMA) monolith. The columns could then be used in the selective capture and desorption of ochratoxin A toxins, ideally for pre-concentration or molecule retention. Chen *et al*. [41] developed a poly(VAL-*co*-HEMA-*co*-EDMA) monolithic column, to which antibodies were immobilized, specifically anti-testosterone. The group was successful in capturing testosterone, however, they noted a low surface availability of antibody on the monolith surface. This was due to the nature of co-polymerization itself [15,39].

4 Chiral Stationary Phases

Guerrouache and co-workers prepared a poly(NAS-*co*-EDMA) monolith, functionalized using a β-cyclodextrin compound, for the separation of flavone enantiomers in both CEC and nano-LC modes [42]. A poly(NAS-*co*-EDMA) monolith was functionalized with aromatic amines (benzylamine, naphthylamine, and phenylbutylamine), to prepare RP columns for CEC separations of alkylbenzenes, anilines, and phenols [42]. The phenylbutylamine modified column showed enhanced separation capabilities as demonstrated through enhanced resolution and aromatic selectivity relative to the other modified columns. The modification of surface NAS groups with propargylamine resulted in pendant alkyne groups, which were further reacted with click chemistry to ultimately produce adamantine columns [45], which were applied to the separation of phenols.

5 Zwitterionic Monolithic Columns

Zwitterionic monomers have been used in co-polymerization of monoliths, as a co-monomer, with applications in HILIC separations [32-35,66], with an excellent review covering the subject recently published [67]. The addition of zwitterionic molecules to a generic monolith was used in the preparation of an immobilized pH gradient. Poly(GMA-*co*-EDMA) monoliths were prepared and subjected to the addition of the zwitterionic groups using epoxide and aldehyde immobilization pathways [59-61]. Direct immobilization of the zwitterionic mixture to the

monolith was also used [60], using primary amino groups within the zwitterion's structure. The columns were used in the CEC separation of proteins from deer antler extract [59], proteins and amino acids [59,61], and cancer cells derived from rats [60]. Dong *et al.* [68] prepared a poly(GMA-*co*-EDMA) monolith, which was subsequently reacted with lysine, to create a zwitterionic stationary phase. The column was used in CEC, where the modification resulted in the generation of EOF. Analytes of different classes were separated under different conditions by varying the pH of the buffer. The column was used successfully in the separation of phenols, aromatic acids, anilines, and basic pharmaceuticals.

6 Photo-grafting Reactions

Where precise spatial location is required (e.g., in the preparation of monoliths within microfluidic devices [7]), the use of reactive sites latent in the monolith structure, such as those described above, does not afford a precise control over functionalization. The availability of surface groups still remains problematic with co-polymerization of functional monomers [15,39]. Graft polymerization involves the growth of polymer chains from the surface of a solid support such as a polymeric monolithic column [7,39]. Photo-initiated grafting procedures from the seminal work of Rånby *et al*. [69-74], were investigated following the innovation of photo-initiated polymerizations in the fabrication of monolithic columns in 1997 [75]. Grafting and photo-initiated polymerizations offer enhanced flexibility relative to co-polymerization of monomers for dedicated chemistries, as the control of morphology is separate from functionalization. Photo-grafting has many uses such as the implementation of a number of different surface functionalities on the surface of a monolithic substrate. Using photo-grafting, a dense polymeric network can be formed from the surface of the polymeric substrate, resulting in a shielding effect of the generic monolith, and resulting in a separate functionality at the surface [31,38,76,77].

Photo initiated graft polymerization techniques are generally performed using either single step or multi-step procedures. For single step grafting, the monomer is present alongside the initiator in solution whilst the column is irradiated. In multi-step grafting, the graft procedure is broken down into two steps. Firstly, the initiator is immobilized to the substrate surface using UV irradiation. In a second step, the column is filled entirely with monomer solution, and the column is exposed to UV irradiation to facilitate grafting. Single step grafting is technically straightforward; however, polymerization occurs not only from the monolith's surface as desired, but also in solution within the pores of the monolith [7]. Solution localized polymerizations form a viscous gel, which may be difficult to remove through washing, resulting in elevated column back pressures.

Stachowiak *et al*. demonstrated improved grafting procedures, wherein a multi-

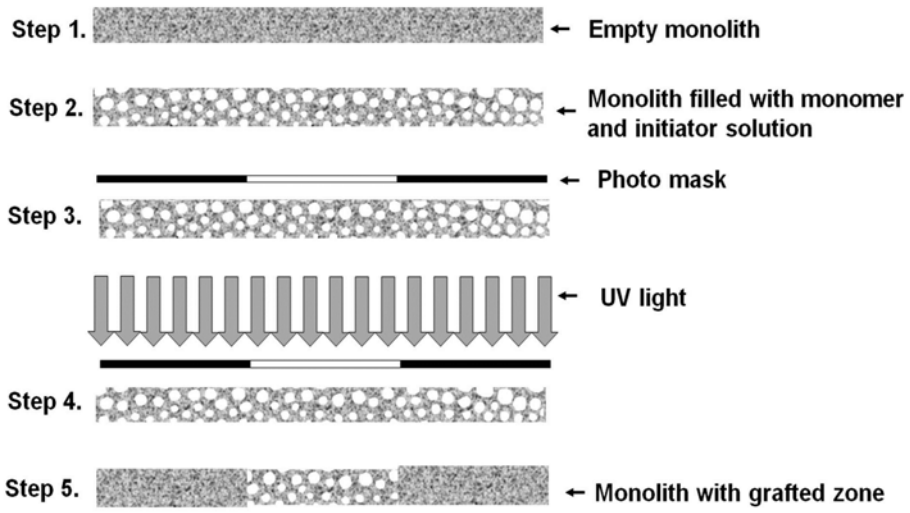

Fig. 1 Scheme of photo-grafting of grafted zone upon on preformed monolithic column

step grafting procedure was developed based on the initiator benzophenone [7]. By using photo induced lysis, the initiator was immobilized to the monolith's inner surface in the absence of monomer, leaving a surface-bound free radical. The UV irradiation procedure causes excitation of the electrons within the polymer, with consequential hydrogen abstraction from the polymer surface. In the absence of monomers, the surface-bound radical binds to the semi-pinacol radical formed by the absorption of UV energy by benzophenone. In the presence of monomers and a secondary exposure to UV energy, the initiator is liberated from the surface, dimerising in solution, exposing the surface-bound free radical for graft chain growth. In using this method, the efficiency of grafting can be enhanced, as fewer monomer molecules are "lost" to solution located chain growth. This technique also reduces the viscosity of free/unreacted materials removed from the column.

6.1 Monolithic Columns Grafted with Ionisable Exchange Groups

Rohr *et al.* developed grafting procedures for monoliths, using "generic" monolithic columns [38,39]. A charged monomer AMPS was grafted to the surface of a poly(BuMA-*co*-EDMA) monolith, wherein effect of grafting time was investigated [39]. The increase in grafting time resulted in a proportional increase in the column pressure drop. This was indicative of the growing dense network of polymer chains, with increasing irradiation time. For CEC, the poly(AMPS) grafts supported electroosmotic flow (EOF). The EOF was found to plateau once a certain density of grafts was achieved, as only those sites at or near to the surface contribute to EOF. The photo-grafting of selective groups in defined areas was also investigated, where reactive VAL groups were grafted to a generic monolith. Fluorescent rhodamine 6G was covalently attached to the VAL sites, enabling a

visual cross-validation of grafted sites. Using a fluorescence assay, an increased fluorescent intensity was observed for a column grafted with the same irradiation intensity, for a longer period of time.

Using a single step graft polymerization with benzophenone as the initiator, Connolly *et al*. grafted META to poly(GMA-*co*-EDMA) monolithic columns in the preparation of grafted anion exchange selectivity [78]. The column was capable of delivering efficiencies of up to 29,500 N m^{-1} (for inorganic anions), higher than the latex agglomerated (quaternary ammonium) monolithic columns described by Hilder *et al*. [48] (26,000 N m^{-1}), which were prepared *via* co-polymerization with modification. In the surface modification of neutral poly(BuMA-*co*-EDMA) monolithic columns, Eeltink *et al*. used single step grafting to which either AMPS or META were grafted [76]. Measurements on pre- and post-modified monoliths have shown that the grafting procedure supported the generation of EOF, on an otherwise neutral monolithic column. Increasing the amount of grafting functional monomer (META) in solution from 0.5 % to 15 % resulted in a ten-fold increase in electro-osmotic mobility. In addition, to the effect of charged monomers, the group investigated the increased retention of hydrophobic analytes in RP CEC by grafting a hydrophobic monomer lauryl methacrylate (LMA). Initially, the column was grafted with an ionisable layer (META), followed by 10 min of grafting of LMA, after which an increase of 53 % in retention factor was observed with RP CEC, in agreement with the findings by Rohr *et al*. [39], and Hilder *et al*. [38].

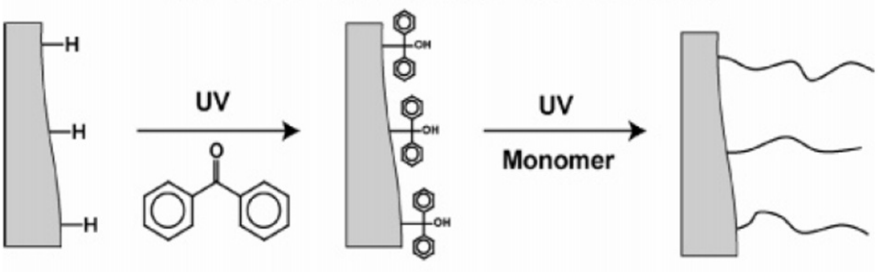

Fig. 2 Grafting of initiator and functional monomer in two discreet steps, upon preformed monolithic column, using benzophenone as photo-initiator [7]. Reprinted with permission

To a neutral poly(BuMA-*co*-EDMA) monolithic column discrete zones of ionized AMPS was grafted [79]. Using a contactless conductivity detector in scanning mode, (scanning C^4D, sC^4D), qualitative information could be obtained wherein the relative density of the grafted zones could be mapped, providing a cross-validation of the grafting procedure. Histidine tagged green fluorescent protein (GFP) was immobilized *via* grafted zones of VAL. Similarly, Gillespie *et al*. studied the optimization of photo-grafting, using poly(AMPS) grafts on poly(BuMA-*co*-EDMA) monolithic columns, whilst using sC^4D as a characterization technique [31]. The zones of grafted charged monomer were subjected to

varying doses of UV energy, and thus resulted in a variation of graft chain polymer density. The detector's response was proportional to the amount of charged monomer grafted in the column; however, it was noted that a plateau in detector response occurred with increased UV doses. This is supported by the observations made by Hilder *et al*. [38,76], wherein the uppermost layer is attributed to the resulting charge density. In addition, the group also demonstrated that a minimum UV dose was required to initiate the photo-grafting step, as doses below 1 J cm^{-2} UV energy (in this case) resulted in no change to the detector response across that zone relative to the baseline monolith.

6.2 Grafted Monolithic Columns for Biomolecule Immobilization.

In order to minimize non-specific binding of a biomolecule to the monolith surface, a neutral hydrophilic monomer [7] may firstly be grafted to the substrate surface [8,13,80-82]. A dual function monolith incorporating a μSPE section followed by an enzymatic reactor was prepared by Peterson *et al.* [77]. A generic hydrophobic monolith was prepared (poly(BuMA-*co*-EDMA)), to which a 20 mm section of monolith was grafted with VAL, for further reaction with trypsin thus creating the enzymatic reactor segment of the monolith. The remaining length of the monolith (hydrophobic) was dedicated to μSPE. In addition, a co-polymerized monolith incorporating VAL was also prepared (poly(EDMA-*co*-HEMA-*co*-VAL)). A higher enzymatic activity was demonstrated by the photo-grafted column demonstrated relative to the co-polymerized monoliths. The effect of time upon the graft density was investigated, and measured by enzymatic activity. Increasing graft time showed a proportional increase in graft density, and pressure drop across the column, within the limits of the experiment, i.e., between 1 and 3 min of grafting. The column was used successfully in the digestion of a peptide, consisting of fluorescent fragments, facilitating the visualisation of the μSPE zone.

A column expressing three segments of differing enzymatic systems, each specifically placed using photo-grafting of the reactive monomer VAL, was prepared by Logan *et al.* [8]. Firstly, the poly(BuMA-*co*-EDMA) monolith was grafted with polyethyleneglycol methacrylate (PEGMA), producing a hydrophilized surface. Three discreet zones of VAL were grafted sequentially in order to immobilize each enzyme (glucose oxidase, horseradish peroxidise, and invertase). In addition, using VAL grafts and green fluorescent protein (GFP), the group determined the use of sequential grafting had little or no effect on the activity of the initially immobilized GFP, and thus immobilized enzymes. The versatility of the photo-masking technique to graft monolithic columns within a single column housing was demonstrated, with a variety of different chemistries *via* subsequent steps.

In an effort to study their resistance to protein adsorption, monomers such

as acrylamide, vinyl pyrrolidinone, 2-hydroxyethyl methacrylate (HEMA), and PEGMA were grafted upon poly(BuMA-co-EDMA) monoliths [7]. Fluorescent bovine serum albumin (BSA) and GFP were flushed across the test columns, where zones of hydrophilic monomer were grafted, resulting in a fluorescent patternization across the column. The protein probes were non-specifically adsorbed on the non-grafted sections of the monolith, while the grafted monomers showed reduced fluorescence intensity (HEMA and PEGMA only). The fluorescence assay provided a qualitative validation of the grafted groups, both in location and ability to resist protein adsorption. The cross-validation of grafted moieties was also probed by Connolly *et al.* [79], wherein poly(VAL) grafts were generated on the surface of poly(BuMA-*co*-EDMA) monolithic columns. The grafts facilitated the immobilization of GFP for fluorescent visualization, and also BSA for sC^4D characterization. Using non-destructive methods of characterisation, such as sC^4D and fluorescence mapping, the spatial location of these groups was visualized.

Photo-grafting was also used by Křenková *et al.* to introduce hydrophilic surface functionality to otherwise hydrophobic monolithic columns [80], similar to the work by Stachowiak *et al*. [7], and Logan *et al*. [8]. Chains of poly(PEGMA) were grafted to poly(GMA-*co*-EDMA) monolithic columns, to reduce the non-specific adsorption of hydrophobic proteins to the surface. In a secondary grafting step, VAL was grafted to facilitate the immobilization of trypsin, and endoproteinase LysC for enzymatic digestion. Prior to the immobilization of benzophenone, the hydrolysis of the epoxide rings of poly(GMA-*co*-EDMA) was required. Using a fluorescence assay, the non-specific adsorption of BSA was visualized before and after grafting. The grafting of poly(HEMA) resulted in a significant decrease in fluorescence intensity. The enzymatic reactor columns (both trypsin and endoproteinase LysC) were used in the successful digestion of a number of proteins, with mass spectra obtained for the digested peptide fragments. Similarly, hydrophilization was repeated by this group [81], for the immobilization of PNGase F, in the analysis of glycoproteins, wherein a multistep grafting procedure was used. A secondary column was used for the subsequent separation of the peptide fragments.

6.3 Monolithic Columns with Immobilized Ligands

Boronate affinity phases are used to selectively extract carbohydrates *via* 1,2- and 1,3-*cis*-vicinal diols which are common to most carbohydrate molecules [82]. In the preparation of boronate phases, Potter *et al*. used two approaches in the preparation of monolith columns (i.e., co-polymerization of GMA vs photo-grafting of poly(GMA) grafts) and compared both methods [82]. The boronate affinity columns demonstrated the retention of ribonucleosides, with an increased

affinity in the photo-grafted column. This was due to an increase in the surface density of grafted polymer chains [31], relative to the sporadic nature of co-polymerized co-monomer [30].

In a separate report, Gillespie *et al.* prepared generic poly(BuMA-*co*-EDMA) monolithic columns, to which zones of *m*-aminophenyl boronic acid were immobilized in two spatially discrete areas *via* poly(VAL) grafts [83]. The column was grafted with PEGMA in order to reduce non-specific binding [7], prior to VAL grafting and attachment of the boronate ligand. Using sC^4D profiling, a study was performed into the pK_a of immobilized boronate ligand following its immobilization using a range of pH buffers. Later, the group performed another investigation of pK_a of aminocarboxylates following immobilization on grafted VAL sites [84]. The pK_a of the immobilized acids were shifted from those reported in the literature for bulk solution. This work demonstrates that photo-grafting can be exploited for the investigation of the fundamental properties of immobilized functional groups.

The composition of monolithic column housing materials limits the use of UV initiating techniques. For *in situ* polymerizations, fused silica capillaries coated in Teflon (PTFE) are generally used for UV-initiated procedures, but diameters greater than 250 μm are not widely available. Polyimide coated capillaries are widely available and cheaper than their PTFE counterparts. Walsh *et al*. used light at a wavelength of 470 nm to initiate the polymerization of poly(styrene-*co*-divinyl benzene) (poly(STY-*co*-DVB)) monolithic columns within PTFE coated capillaries [85]. Later, an in-house prepared spiropyran monomer was grafted to the surface of poly(BuMA-*co*-EDMA) monolithic columns in polyimide coated capillaries, using light initiation at 660 nm [86].

The preparation of chelate immobilized monolithic columns has been so far restricted to the ring opening reactions of GMA, in GMA supported monoliths, but few rely on surface grafting [13,54,84]. Moyna *et al.* prepared poly(LMA-*co*-EDMA) monolithic columns, with subsequent poly(VAL) grafts [87]. Chelating ligands such as IDA and acetyl-iminodiacetic acid were immobilized *via* the poly(VAL) grafted chains. The resulting columns demonstrated efficiencies lower than 5,000 N m^{-1}; however, enhanced selectivity was evident. In a second report [88], Moyna *et al*. grafted poly(LMA-*co*-EDMA) monolithic columns with the epoxide containing GMA monomer, which was subsequently used for IDA immobilization. The column was used in the separation of transition metals in seawater samples; however, the efficiency although improved from poly(VAL) grafts [88], was still relatively low, at 5,070 N m^{-1}.

6.4 Monolithic Columns Grafted with Heterogeneous Grafting Energies.

The density of the graft in UV initiated grafting can be altered using UV energy, time of irradiation, and concentration of the monomer/initiator. As first described by Rohr *et al*. [39], the use of filters could produce a grafted gradient of functional monomer, where the grafting efficiency increased with increasing light exposure for grafting [39,89]. Pucci *et al*. prepared poly(BuMA-*co*-EDMA) monolithic columns, which were later modified with AMPS using a number of different methods in order to create a grafted gradient of functionality upon a homogeneous monolith, as described in Table III [10]. Using single step modification, three methods of modification were investigated. The successful method included a polymer film, to reduce the intensity of the UV source, whilst a moving shutter changed the temporal exposure of the column. By reducing the intensity of light, a longer irradiation time could be used, resulting in a lower chance of experimental error and increased reproducibility. From the three columns prepared, the greatest column efficiency was achieved, with 85,900 N m^{-1} in the CEC separation of salicylic acid and acetylsalicylic acid.

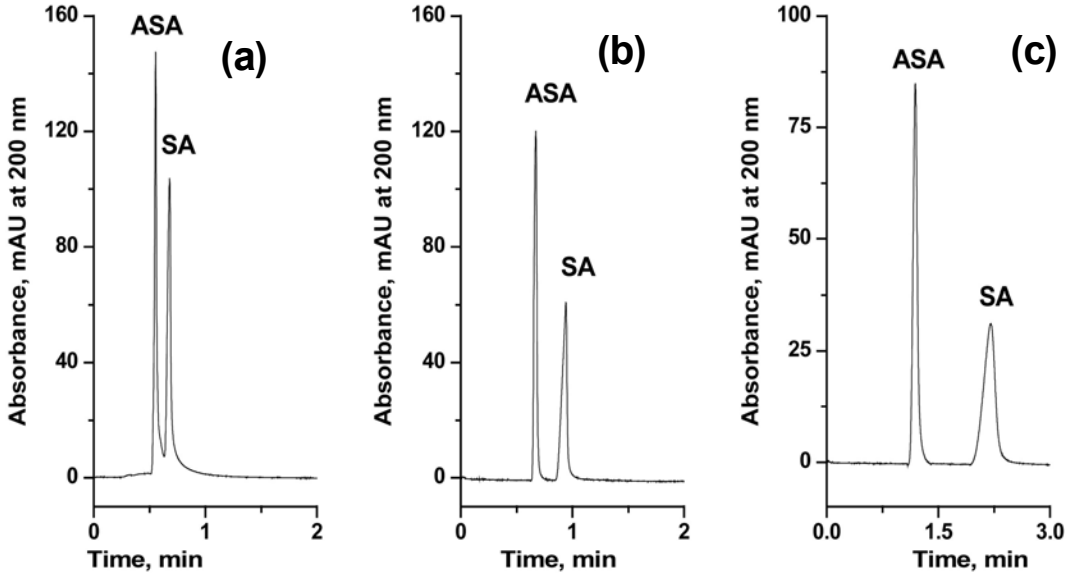

Fig. 3 Chromatograms generated on isotropic column (a), gradient prepared using moving shutter (b), and gradient produced using neutral density filter (c). Analytes ASA = acetylsalicylic acid, SA = salicylic acid. Mobile phase 80:20 ACN:10 mM phosphate buffer pH 2.5. Separation voltage = 25 kV, electrokinetic injection at 5 kV for 5 s. UV detection at wavelength of 200 nm [10]

Currivan *et al*. prepared gradients of grafted functionality of AMPS upon a poly(BuMA-*co*-EDMA) monolithic column, using a commercially available neutral density gradient filter [11], similar to the work performed by Pucci *et al.* [10]. The distribution of the gradient was also characterized using sC^4D profiling,

which provided qualification of the gradient indirectly. However, the attenuation of the gradient filter was too great due to an excessive absorbance. Currivan *et al*. later produced a grafted stationary phase gradient which was fabricated using commercially available cyclic olefin copolymer (COC) films [13]. The gradient of functionality was prepared upon otherwise neutral poly(LMA-*co*-EDMA) monoliths, some of which had been subjected to hydrophilization with PEGMA grafting. The ease in tailoring the material to the absorbance range made it an attractive method for UV light attenuation. The columns were modified using poly(VAL) for IDA immobilization, and poly(SPM) grafted chains. The SCX column was used in the separation of lysozyme and transferrin, and by changing the direction of flow through the gradient column, a decrease in peak width of 26 % was observed, demonstrating peak focusing ability of such technology.

Table III Reactions used in production of photo-grafted stationary phases

Monolith	Reaction	Graft energy	Time	Ref.
Poly(BuMA-co-EDMA)	Single and sequential two step grafting of monomers.	500 W	60* min	[7]
Poly(BuMA-co-EDMA)	Sequential grafting of PEGMA and VAL.	500 W	60* min	[8]
Poly(BuMA-co-EDMA)	Single step grafting of gradients of AMPS.	500 W	1 to 10 min	[10]
Poly(BuMA-co-EDMA)	Single step grafting of gradients of AMPS.	3 J cm^{-2}	N/A	[11]
Poly(BuMA-co-EDMA)	Sequential grafting of PEGMA, VAL, and SPM.	1 J cm^{-2} each step	N/A	[13]
Poly(BuMA-co-EDMA)	Single step grafting of AMPS.	0.25 to 7 J cm^{-2}	N/A	[31]
Poly(BuMA-co-EDMA)	Single step grafting of AMPS and butyl acrylate.	500 W	0.5 to 1.5 min	[38]
Poly(BuMA-co-EDMA)	Single step grafting of AMPS and VAL.	500 W	30 min	[39]
Poly(BuMA-co-EDMA)	Single step grafting of META and butyl acrylate.	1.4 mW cm^{-2}	3 min	[76]
Poly(BuMA-co-EDMA)	Single step grafting of VAL for dual function monolith.	15 mW cm^{-2}	3 min	[77]
Poly(GMA-co-EDMA)	Single step grafting of META.	0.252 J cm^{-2}	N/A	[78]
Poly(BuMA-co-EDMA)	Single step grafting of AMPS and VAL.	N/A	60, 30 min respectively	[79]

Table III – Continued

Monolith	Reaction	Graft energy	Time	Ref.
Poly(GMA-co-EDMA)	Sequential grafting of PEGMA and VAL.	12 mW cm^{-2}	4* 1 to 6 min (VAL)	[80]
Poly(BuMA-co-EDMA) and Poly(GMA-co-EDMA)	Sequential grafting of PEGMA and VAL.	N/A	4*, 2 min	[81]
Poly(BuMA-co-EDMA)	Sequential grafting of PEGMA and VAL.	3* J cm^{-2}	N/A	[83]
Poly(BuMA-co-EDMA)	Single step grafting of VAL.	3 J cm^{-2}	N/A	[84]
Poly(BuMA-co-EDMA)	Single step grafting of spiropyran monomer using VIS LED at 660 nm.	0.5 cd	2 min	[86]
Poly(LMA-co-EDMA)	Multi step grafting of VAL.	1* J cm^{-2}	N/A	[87]
Poly(LMA-co-EDMA)	Multi step grafting of GMA.	1* J cm^{-2}	N/A	[88]

* for each grafting step

7 "Click"chemistry

A technique used originally in organic synthesis has become an emerging technique for *in situ* post polymerization modification of monolithic columns [90]. The technique has been used on silica, and hybrid silica-polymeric monolithic columns [90-95]; however, this review is limited to polymeric monolithic columns. Click reactions include alkyne-azide, thiol-ene, and thiol-yne reactions [90], and may be conducted using elevated temperatures or at ambient room temperature. Unlike many polymer graft reactions, wherein molecular oxygen must be removed prior to polymerization, click modification reactions occur in the presence of water and oxygen [90]. Some reactions are completed in the presence of an initiator such as AIBN or DAP. In these techniques, a surface thiol is required. Preparation methods can be seen in Table IV.

7.1 Thiol-ene Click Reactions in Monolithic Column Functionalization

Preinerstorfer *et al.* prepared poly(GMA-*co*-EDMA) monolithic columns for chiral selector modification [96]. The column was successfully used in the separation of *N*-3,5-dinitrobenzoylleucine enantiomers; however, relatively low efficiencies

were obtained (figures not published), and attributed to the inherent macro-porosity of the monolithic porous structure. Later, Preinerstorfer *et al*. prepared poly(GMA-*co*-EDMA) monoliths for thiol functionalization [52]. The available content of epoxide groups was only 10 %, due to the incorporation of the large majority of epoxides within the monolith structure, similar to that reported by Sýkora *et al*. [46]. The column was applied to the separation of enantiomers of mefloquine *tert*-butylcarbamate (>47,000 N m^{-1}).

Lv *et al.* produced poly(isocyanoethyl methacrylate-*co*-methyl methacrylate-*co*-EDMA) monoliths (poly(IEM-*co*-MMA-*co*-EDMA)) to facilitate modification using hydroxyl, amine, or thiol functionalities [97]. During the modification reactions, the group noted the effect of steric hindrance on the yield of the preferred functionality; the longer the alkyl chain length of the respective modifiers, the lower was the ligand density. In a separate report, using thiol-ene reactions in both UV and thermal initiated addition reactions, a RP column (LMA) and a HILIC column (MEDSA) were produced, by gold nano-particle functionalization [98]. The UV initiation method outperformed the thermal initiation, with a ten-fold increase in plate count for uracil (3,000 N m^{-1} to 30,000 N m^{-1}, respectively), and a lower pressure drop across the column. A zwitterionic column was prepared using a betaine type methacrylate providing an efficiency of 19,000 N m^{-1} for guanosine, in the separation of nucleotides, greater than the column prepared by direct co-polymerization of MEDSA and EDMA [32].

Tijunelyte *et al.* [99] prepared a poly(NAS-*co*-EDMA) monolithic column, modified with allylamine, after which thiolated oligoethylene glycol (OEG) was grafted to the column *via* surface alkene groups, i.e., thiol-ene clicking. A yield of 50 % was achieved, due to the large size of OEG units, similar to that reported by Lv *et al.* [97]. The column was used for the separation of DMF, acrylamide, and toluene and showed dual retention mechanisms dependent on the percentage of organic modifier in the mobile phase, typical of HILIC columns. Additionally, a RP column was prepared using the thiol-ene click pathway, resulting in a C_{18} bonded stationary phase.

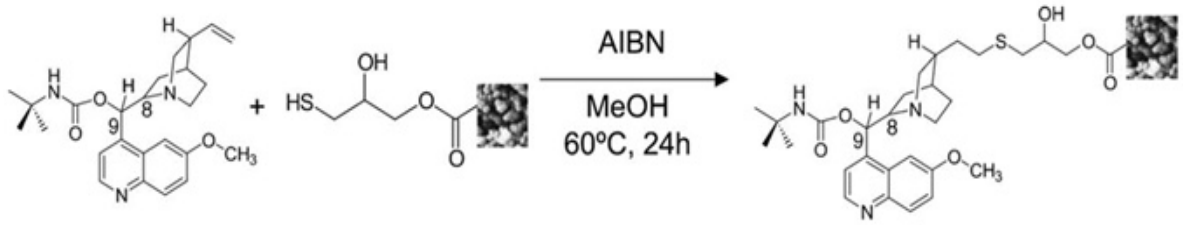

Fig. 4 Scheme of thiol-ene click reaction, with radical (AIBN) addition of *O*-9-tert-butylcarbamoylquinine to available surface bound thiol groups. Reprinted from [96]. Copyright (2004), with permission from Elsevier

Table IV Reactions used in "click" chemistry immobilization strategies

Monolith	Reaction	λ nm	Temperature °C	Time	Ref.
Poly(NAS-*co*-EDMA)	Propargylamine modification to produce surface alkyne. Click grafting of 1-adamantanethiol using UV irradiation at 313 nm, using darocure as initiator.	313	N/A	30, 60, 120, and 180 min	[45]
Poly(GMA-*co*-EDMA), Poly(GMA-*co*-MMA-*co*-EDMA), Poly(GMA-*co*-HEMA-*co*-EDMA)	Surface sulfhydryl groups clicked with (*S*)-*N*-(4-allyloxy-3,5-dichlorobenzoyl)-2-amino-3,3-dimethylbutanephosphonic acid in the presence of AIBN initiator.	N/A	60	24 h	[52]
Poly(GMA-*co*-EDMA)	Surface thiol groups clicked with *O*-9-tert-butylcarbamoylquinine in the presence of AIBN.	N/A	60	24 h	[96]
Poly(IEM-*co*-MMA-*co*-EGDMA)	Monoliths modified with 1-octanol, 1-decanol, 1-dodecanol, 1-octadecanol, *n*-decylamine and 1-decanethiol.	N/A	60	N/A	[97]
Poly(GMA-*co*-EDMA)	Monolith thiolated with cysteamine, with cleavage of the disulfide thus exposing thiols. MEDSA or LMA clicked using either heat or UV initiation.	360	80	N/A	[98]
Poly(NAS-*co*-EDMA)	Allylamine modified monolith surface reacted with 1-octadecanethiol in the presence of AIBN and UV initiation.	365	N/A	4 h	[99]
Poly(propargyl methacrylate-*co*-EDMA)	Click addition of 1-azidooctane and 1-azidooctadecane using a Cu(I) catalyst.	N/A	30	120 h	[100]
Poly(GMA-*co*-EDMA) and poly(VBC-*co*-DVB)	Azide modified surfaces clicked with 1-decyne using a Cu(I) catalyst.	N/A	30-60	48 h	[101]
Poly(3-(Trimethoxysilyl) propyl acrylate-*co*-propargylacrylate-*co*-AMPS-*co*-TRIM-*co*-PETRA)	Active surface modified with cinnamidyl azide or 6-azido-6-deoxy-β-cyclodextrin.	N/A	84	16 h	[102]

7.2 Thiol-yne Click Reactions in Monolithic Column Functionalization

Adamantanethiol was clicked to the surface of propargylamine modified poly(NAS-*co*-EDMA) monoliths, using UV initiation at 313 nm [45]. A solution of 0.5 M 1-adamantanethiol (in MeOH) and the initiator darocure (10 % w.r.t.

adamantanethiol concentration) were pumped across the column prior to the modification reaction. Sun *et al.* produced a monolithic column, with direct co-polymerization of the alkyne monomer propargyl methacrylate and EDMA [100]. The pendent alkyne functionalities were further reacted with in-house synthesized azides (1-azidooctane, 1-azidooctadecane). A number of proteins were separated using the two modified columns. In a later report, Sun *et al*. used poly(GMA-*co*-DVB) and poly(vinylbenzyl chloride-*co*-DVB) (poly(VBC-*co*-DVB)) monoliths for alkyne cyclo-addition [101]. Increasing the reaction temperature resulted in an increase in yield from ~30 % to 100 %. Salwinski *et al*. produced poly(propargyl acrylate-*co*-pentaerythritol triacrylate-*co*-AMPS-*co*-trimethylolpropane trimethacrylate) monolithic columns for applications in CEC [102]. The columns were modified *via* pendent alkyne functionalities on the monolith inner surface. Using 6-azido-6-deoxy-β-cyclodextrin and cinnamyl azide the columns were modified, after which the EOF decreased. Due to the formation of additional layers on the monolith's inner surface, resulting in a shielding effect for AMPS, which is the source of EOF, also noted in graft polymerization reactions [31,38].

8 Thermally Initiated Graft Chain Growth

Thermally initiated graft polymerization was first reported for monolithic columns in 1997 by Peters *et al*. [40]. A monolithic column was produced with thermally grafted polymer chains, which exhibited thermal responsive behaviour. Two types of thermal responsive composites could be formed; a thermal gate wherein all flow was blocked through the column, or a thermal valve which can control flow rate. With the expansion and contraction of the polymer at different temperatures, the resulting change in functionality could be exploited with selective elution of hydrophilic analytes at high temperatures, and elution of hydrophobic analytes at a lower temperature.

The charged monomer AMPS was grafted to an otherwise neutral poly(GMA-*co*-EDMA) monolithic column, in the work performed by Viklund *et al.* [103]. By cooling the column and with extensive washing, the polymerization was terminated. The column was used for the separation of proteins (myoglobin, chymotrypsinogen, lysozyme), with an efficiency of 6,700 N m^{-1} for lysozyme. Poly(TRIM) monoliths were thermally grafted with the zwitterionic monomer MEDSA [104], which retained basic proteins when loaded from pure water.

Other thermally initiated polymerizations used in post-polymerization modification, known as living polymerizations, have been employed to introduce new surface functionality and to improve the fine control of porous properties in polymer monolithic columns. A polymerization that can restart chain growth upon the addition of a new monomer is called "living". During the polymerization process, the stable free radicals are combined with the growing polymer chains.

A functional monomer can then be added to the structure, and with the activation of latent dormant radicals, surface localized grafting can proceed upon the application of temperature [105]. This may provide a method for controlling the phase separation, resulting in more homogeneous monolithic column [17].

There are numerous types of living polymerization approaches such as nitroxide mediated polymerization [105,106], ring opening metathesis polymerization (ROMP) [100,101], atom transfer radical polymerization (ATRP) [109,110], and reversible addition-fragmentation chain transfer polymerization (RAFT) [111,112]. Some of these methods have been used in the fabrication of cross-linked gels [109], polymer beads [110], and polymer monoliths [105,113]. Temperatures above 100 °C are generally used, while termination of the growing polymer is mediated by decreasing the temperature [113]. This discussion is limited to the surface modification of porous polymer monolithic columns.

Peters *et al*. used 2,2,6,6-tetramethylpiperidyl-1-oxyl (TEMPO) mediated polymerizations for the subsequent grafting of the resulting polymer [114]. Either HEMA or VBC were grafted to the column, in the presence of initiators such as benzoyl peroxide (BPO) and TEMPO. Meyer *et al*. prepared poly(styrene-*co*-DVB) (poly(STY-*co*-DVB)) monolithic columns [105]. Using a simplified grafting reaction, wherein the monomer (AMPS) and solvent (cyclohexanol) were only present in the column, with the surface latent initiators mediating the reaction, the graft polymerization proceeded at an elevated temperature of 110 °C for 168 h. Viklund *et al*. prepared poly(STY-*co*-DVB) monolithic columns using BPO and a stable free radical [113]. The hydrophobic poly(STY-*co*-DVB) column was grafted with a hydrophilic monomer (HEMA) using the latent surface radicals. The monomer SPM was also grafted to the column, using a solution of monomer in DMSO. The hydrophilized column (HEMA graft) showed a significantly reduced retention, and a decrease in pore volume of 11.5 % following grafting. The SPM grafted monolith was used in the cation exchange of proteins, namely myoglobin, cytochrome C, and lysozyme.

9 Hypercross-linking

Hypercross-linking occurs when the precursor material or substrate is solvated in a thermodynamically good solvent, where a cross-linking reaction locks the polymer chains in a state similar to that in the solvated state [115]. Following the removal of the solvent, the remaining structures are the new pores within the material, adding significantly to surface area in monolithic columns. The origins of hypercross-linking are with Davankov *et al*. from the 1970's to the early 2000's [115-120]. The hypercoss-linking method is advantageous as generic monolithic columns lacking in meso-pores, with which relatively low specific surface area (usually < 10 m^2 g^{-1} [2]) can be improved upon, by a straight forward post-

polymerization modification. Urban *et al*. highlighted the main theoretical considerations of hypercross-linking [121]. The extent of DVB in the monomer precursor and the ratio to VBC controls the degree of cross-linking, as well as the ratio of VBC to STY, which controls the distance between reactive chloromethyl sites and the extent of hypercross-linking. Hypercross-linking was performed in monolithic columns, using poly(STY-*co*-VBC-*co*-DVB) monolithic columns [121]. The total porosity of the monolith was not altered, due to the minimal mass of the cross-linked moieties; however, the surface area of the monolith was increased from 29 $m^2 g^{-1}$ to 663 $m^2 g^{-1}$, twice the surface area of silica monolithic columns [122]. The isocratic separation of small molecules, prior to hypercross-linking, showed little efficiency with the unmodified column. Following the cross-linking reaction, the separation efficiency for alkylbenzenes performed under the same conditions, increased to 73,000 N m^{-1}. In the gradient separation of proteins, however, the efficiency was significantly reduced, as the introduction of meso-pores resulted in the higher impedance of mass transfer of the proteins. The group investigated the effects of the variables (temperature, time) upon the cross-linking [123]. For reaction times of up to 2 h, the surface area increased dramatically; however, no significant changes in surface area were obtained with increased reaction times. The column was successfully used in three separation types; small molecules (alkylbenzenes), biomolecules (a tryptic digest of the cytochrome C protein), and size exclusion chromatography (toluene with polystyrene standards of varying molecular size). A column temperature of 80 °C produced columns with greatest efficiency, with lower resistance to mass transfer when compared to lower column temperatures. Separations performed with the column hypercross-linked at 90 °C for 2 h produced plate heights in excess of 80,000 N m^{-1}.

Chen *et al*. produced hypercross-linked columns for applications in CEC [124], using the procedure outlined by Urban *et al*. [123]. Poly(STY-*co*-DVB-*co*-VBC) and poly(4-methyl styrene-*co*-DVB-*co*-VBC) (poly(MST-*co*-DVB-*co*-VBC)) columns were prepared for hypercross-linking, after which the poly(STY-*co*-DVB-*co*-VBC) monolith demonstrated a 34 fold increase in EOF, while the hypercross-linked poly(MST-*co*-DVB-*co*-VBC) monolith exhibited a 21 fold increase, relative to the unmodified generic monolith. The increase in EOF was attributed to the increase in surface area in the column due to the formation of new meso-pores. Another use of the column design by Urban *et al.* [123] was demonstrated by Teisseyre *et al.* who coupled NMR to on-line micro-HPLC separations [125].

The technique of hypercross-linking has recently been extended by Škeřiková *et al*., wherein following hypercross-linking a monomer (MEDSA) was thermally grafted to the resulting porous structure [126]. The surface modification demonstrated an excellent stability, with a % RSD no greater than 4 %, for retention times following 500 injections. The modified column demonstrated a dual retention mechanism, dependent on the amount of water in the mobile phase

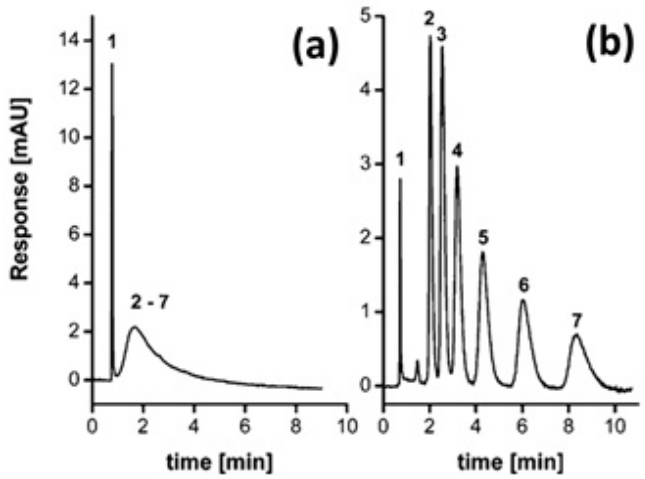

Fig. 5 Separation of uracil and alkylbenzenes using (a) generic precursor poly(STY-co-VBC-co-DVB) monolithic column, and (b) hypercross-linked counterpart. Separation conditions: 80 % ACN, flow rate 1.5 μl min^{-1}, UV detection at 254 nm. Peaks: uracil (1), benzene (2), toluene (3), ethylbenzene (4), propylbenzene (5), butylbenzene (6), and amylbenzene (7) [121]. Reprinted with permission. Adapted with permission from [121]. Copyright (2010) American Chemical Society

Table V Hypercross-linking used in the preparation of enhanced surface area polymeric monolithic columns

Monolith	Reaction	Temperature °C	Time h	Ref.
Poly(STY-*co*-VBC-*co*-DVB)	Solution of $FeCl_3$ in DCE flushed across column (held in ice for 2 h).	80	24	[121]
Poly(styrene-*co*-VBC-*co*-DVB)	Solution of $FeCl_3$ in DCE flushed across column (held in ice for 1 h).	80	24	[123]
Poly(STY-*co*-VBC-*co*-DVB) and poly(MST-*co*-VBC-*co*-DVB)	Solution of $FeCl_3$ in DCE flushed across column (held in ice for 2 h).	90	2	[124]
Poly(STY-*co*-VBC-*co*-DVB)	Solution of $FeCl_3$ in DCE flushed across column.	90	2	[125]
Poly(STY-*co*-VBC-*co*-DVB)	Solution of $FeCl_3$ in DCE flushed across column. Thermal grafting of MEDSA using 4,4´-azobis(4-cyanovaleric acid) (8 h at 70 ˚C).	90	2	[126]
Poly(MST-*co*-VBC-*co*-DVB)	Hypercross-linked monolith brominated to support cysteamine modification (microwave assisted, 30 min). Disulfide bonds cleaved. Pendent thiols modified with AuNPs.	90	4	[127]

(φ H_2O), characteristic of such columns [32-35,37]. Hypercross-linking may be combined for further modification, for example with nano-particles, for the

preparation of novel stationary phases [127]. A comparison of hypercross-linking reactions can be seen in Table V.

10 Scaffold Monolithic Columns

The overall aim of post-polymerization modifications is to improve column efficiency by introducing a higher proportion of functional groups per unit area of the column, either through an increase in available surface area or by the addition of polymer chains. Short polymerization times have been recently reported to have increased efficiencies in the separations of small molecules [128-134]. Columns packed with superficially porous silica particles have been prepared by Jandera *et al*. [128,129], with additional polymerization for fast separations. The column packing was added to the monolith precursor monomer solution, and sonicated until the pores of the particles were also filled with the monomer solution. The suspension was filled into the column housing and polymerization preceded at 70 °C for 24 h. Relative to the monolith only columns, the hybrid columns demonstrated lower permeabilities with increased efficiencies. Later, Jandera *et al*. [129] prepared monolith-particle hybrid columns, using pre-packed capillary columns containing silica particles with bonded C_{18} or aminopropyl moieties. Capillary columns packed with particles (3 or 5 μm) were filled with monolith monomer precursor solution, and polymerization was performed *in situ*, within the pre-packed column. The polymer acted as an adhesive, holding the particles in place. The resulting hybrid columns showed enhanced separation ability, relative to their unmodified counterparts, and decreased permeability following the introduction of monolithic material. The use of a scaffold in the preparation of monolithic media has also been presented in the production of ordered structure monolithic columns [130].

Greiderer *et al*. demonstrated that shorter polymerization times may result in improved column efficiencies, when preparing styrenic monolithic columns [131]. Trojer *et al*. also demonstrated the increase in meso-pore population with decreased polymerization times, again with styrenic monomer systems [132]. Nischang and co-workers investigated the time of polymerization upon methacrylic monolithic columns, with the time required for polymerization ranging between 30 min and 48 h [133]. The group discovered that a minimum of 30 min was required in the formation of a coalesced rigid monolith. Following their work on methacrylic monolithic columns, the group moved to styrenic monolithic columns for further studies [134]. Combining shortened polymerization times, and a secondary polymerization has recently been investigated by Currivan *et al*. [135]. Methacrylate monolithic columns were prepared, and used as scaffolds, with a secondary polymerization step of betaine type monomer, MEDSA. Separations for these columns have so far yielded efficiencies of up to 55,000 N m^{-1} for thiourea (95 % ACN mobile phase).

11 Nano-particles and Nano-architectures

Nano-particles are characterised by their high volume to surface area ratio [136], and as such, can also be used to increase the surface area of polymeric monolithic columns *in situ*. Nano-particles and nano-structures can be introduced to monolithic columns primarily through two routes; firstly, the addition of the nano-particle or nano-structure to the monolith precursor solution prior to polymerization (encapsulation) [137-145], or secondly, by immobilization on the monolith surface [9,146-156]. Three recent excellent reviews on nano-particle modifications have been recently published, dealing with both nano-particle modified stationary phases for biomolecule immobilizations [29], and nano-particle stationary phases for columns in HPLC separations [27,28]. An SEM micrograph is presented in Figure 6, demonstrating the immobilization of nano-particles to a polymeric monolithic column.

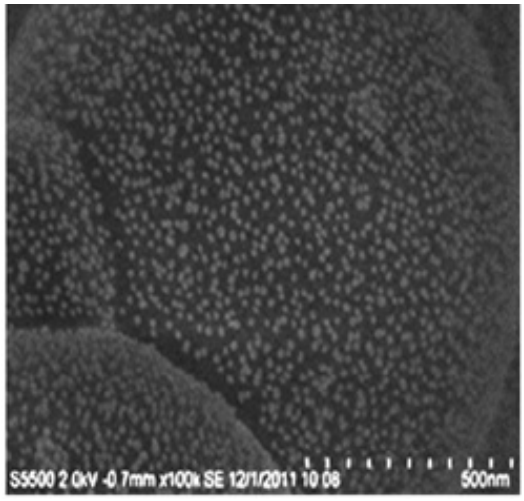

Fig. 6 FE-SEM micrograph of poly(LMA-co-EDMA) monolith with aminated poly(GMA) grafted chains to support AuNP immobilization. Reprinted from [9]. Copyright (2013), with permission from Elsevier

As observed with the co-polymerization of functional monomers, problems associated with encapsulation of the nano-architecture within the polymer globule such as inaccessibility, and changes with monolith structure brought on by the additional nano-architectures [138], can be detrimental in the finite control of column morphology. The current review is limited to post-polymerization modifications, and so encapsulation will not be discussed further. In the surface modification with nano-architectures, a functional group responsible for nano-particle attachment is co-polymerized into the monolith, or a polymeric network is formed from the surface of the monolith substrate. In the case of co-polymerization of the functional monomer, the accessibility to the surface may be hindered (see Section 2.1), and the resulting coverage may be low, limiting the

effect of the nano-architecture. A summary of surface modifications using nano-materials can be seen in Table VI.

11.1 Polymeric Nano-particles

The use of quaternary ammonium expressing latex nano-particles was reported by Hilder *et al*. [48] and later by Hutchinson *et al*. [30,49,50]. Using a single step co-polymerization, the functional monomer AMPS was used to generate surface sulfonic acid groups, for subsequent modification with latex nano-particles. The nano-particles were prepared with quaternary ammonium exchange functionality, and by utilising coulombic interactions with the surface sulfonic acid groups the immobilization could be supported. The anion exchange nano-particles (60 nm diameter) were flushed across the column to facilitate coating. One of the advantages noted by the group was the increase in surface area, which improved by approximately 30 %, relative to the unmodified column. This column type has been used successfully in the separation of carbohydrates [48], inorganic ions in sea water and in milli Q water [50].

11.2 Gold Nano-particles

The immobilization of nano-particles has been used to facilitate the addition of a functional group [146,147], and to provide a substrate to facilitate interchangeable surface functionality [148]. Procedures for gold nano-particle (AuNP) immobilization vary from latent reactive surface groups such as those generated by co-polymerization of reactive monomers (e.g., GMA, NAS) [149], and the grafting of functional groups capable of supporting aminated or thiolated groups for immobilization [9,146,150,151]. A method for the preparation of AuNPs was described by Frens *et al*. [157], in which the concentration of reducing agent controlled the resulting nano-particle diameter. Generally, the AuNPs are formed *in vitro* prior to addition to a preformed monolithic column [9,146,151]. Alternative methods can also be used such as the mixing of the AuNP suspension with a reducing agent directly before entering the column, i.e., by use of a T-piece [148,150].

The grafting of reactive monomers VAL or GMA was used in numerous studies [9,146,151] in order to produce the typical surface for AuNP immobilization. Connolly *et al.* investigated aminated and thiolated surfaces for AuNP immobilization; amination provided uniformly spaced nano-particles, whereas a thiolated surface produced sporadic aggregates of nano-particle clusters [151]. Higher gold wt % content on the monolith surface was generated *via* the aminated pathway, relative to previously reported thiolated methods [148,150]. A

hypercross-linked column was produced by Lv *et al*.; it incorporated the use of both surface thiol groups and a secondary immobilization of amine groups (polyethyleneimine, PEI) [127]. The thiol layer was responsible for the primary loading of 20 nm AuNPs, whilst the secondary aminated layer supported the immobilization of 10 nm AuNPs. Ultimately, the column demonstrated both an increase in available surface area, and an increase in separation efficiency. As the total content of gold was lower than previously reported columns [148,150], this increase in efficiency may have been due to the hypercross-linking step alone.

Once AuNPs have been immobilized, the surface can be used for additional functionalization. For example, Cao *et al*. [148] have used the AuNP surface to produce switchable surface chemistries, by exploiting the interaction between thiols and gold. A number of molecules were used to produce RPLC functionality, ion exchange functionality, and charged groups for CEC separations. Each stationary phase demonstrated the separation ability and the stability of the AuNP coating, using model analytes of three peptides: ribonuclease A, cytochrome C, and myoglobin. As the coating is also temperature sensitive, as described by Xu *et al.* [150], the AuNP surface can be regenerated using temperatures of above 80 °C and water to remove thiols.

Immobilized AuNPs can also be utilised in the preparation of bio-affinity phases. Alwael *et al.* [146] produced poly(EDMA) monoliths within the confines of polypropylene pipette tips, which were subsequently modified with AuNPs. Following multiple fabrication steps, the monolith ultimately expressed a lectin which could be selectively used to extract glycoproteins from complex mixtures. The pipette tip demonstrated an extraction efficiency of up to 37 %, and the recovery efficiency of 86 % to 100 %. A segmented AuNP monolith was produced by Currivan *et al*. [9], wherein, similar to the photo-grafting work performed by Logan *et al*. [8], a column expressing two discreet zones was prepared. A section of column dedicated to AuNP immobilization was followed by a RP zone, and using an elution technique described by Xu *et al*. [150], cysteine containing peptides could be extracted and separated within a single column. In a recent report, Zhang *et al*. [147] produced an AuNP modified monolithic enzymatic micro-reactor, within a capillary for CE. The AuNPs were modified with α-glucosidase, and used in the screening of α-glucosidase inhibitors in a range of natural products.

11.3 Other Metallic Nano-particles

In addition to AuNPs, metallic or metal oxide nano-particles are also available, including nickel-cobalt [141], and titanium dioxide [142,143]. Silver nano-particles (SNPs) were trapped in the crevices of a hydrophilic monolithic column poly(GMA-*co*-trimethylolpropane triacrylate). The SNP modified column was

effectively used as a detection element for surface enhanced Raman spectroscopy (SERS) [144]. Iron oxide nano-particles have been used to prepare monolithic media in both column [136] and pipette format [152]. For modification with iron oxide nano-particles (IONPs), Křenková *et al*. prepared poly(GMA-*co*-EDMA) monolithic columns [136]. Once modified, the IONP column was applied to the enrichment of phosphopeptides (α-casein, and β-casein tryptic digests), with MS detection. The monolithic column demonstrated higher selectivity when compared to commercially available TiO_2 pipette tips. Using ATP, the binding capacity of the monolith was found to be 47.4 mg ml^{-1} column volume (86 μmol ml^{-1} column volume). Later, this technology was extended to the confines of a pipette tip [152]. The quaternary ammonium monomer META was grafted to the column, enabling the immobilization of IONPs. The pipette tip was used in the enrichment of phosphopeptides (α-casein and β-casein), and was compared to commercially available TiO_2 tips. Again, the IONP modified monolithic tip exhibited superior selectivity.

Li *et al*. [153] immobilized γ-Al_2O_3 nano-particles to a poly(*N*-isopropylacrylamide-*co*-GMA-*co*-EDMA) monolith. The material was used in the polymer monolith micro-extraction (PMME) procedure of Sudan dyes in red wine samples. The dye was extracted using PMME, and using particle packed C_{18} column the analytes were detected. The resulting extraction efficiency ranged from 84 to 116 %. For the analysis of the dyes, the PMME method was compared with an unmodified monolith and direct HPLC analyses. A four-fold increase in peak area for the nano-particle modified column relative to the polymer monolith alone was observed, and an eight-fold increase relative to direct HPLC analysis, thus demonstrating the positive effect of the nano-particles.

Composite nano-particles consisting of metals have also been immobilized to generic monolithic columns [154]. Floris *et al*. [154] prepared poly(BuMA-*co*-EDMA) columns, grafted with VAL or GMA to support column amination. Following the modification, platinum/palladium nano-flowers (NFs), consisting of a platinum core to which palladium "petals", were immobilized to the inner surface of the aminated polymeric monolith. The columns were evaluated using chromatography *via* anion exchange, performed before and following modification. The NF modified column was also successful in catalysis, in the catalytic reduction of Fe(III) to Fe (II), and catalytic oxidation of NADH to NAD^+. The immobilized NFs demonstrated an excellent reproducibility, and stability in the presence of strong reducing agents such as sodium borohydride.

11.4 Nano-structures

Nano-structures have also been used in the preparation of monolithic columns, and encompass carbon nano-structures (e.g., multi-walled carbon nano-tubes,

Table VI Reactions used in preparation of nano-agglomerated polymeric monoliths

Monolith	Reaction	Temperature °C	Time	Nano-particle type	Ref.
Poly(BuMA-*co*-EDMA) and poly(LMA-*co*-EDMA)	Photo-grafted zones of monomers suitable for amination, supporting AuNP immobilization. Dual function monolith.	Room temperature (RT)	N/A	20 nm Au	[9]
Poly(GMA-*co*-EDMA) and poly(STY−*co*-DVB)	Poly(STY-*co*-DVB) chlorosulfonic acid in dry dichloromethane was used for sulfonation. Poly(GMA-*co*-EDMA) sulfonation was performed in three techniques: (i) 4-hydroxybenezene-sulfonic acid and triethylamine, (ii) thiobenzoic acid and triethylamine (thiol groups were oxidised by pumping a solution of *tert*-butylhydroperoxide). (iii) Sodium sulfite and tetra-*n*-butylammonium hydroxide.	60, 60, 70, respectively	20 h each	65 nm Latex	[30]
Poly(BuMA-*co*-AMPS-*co*-EDMA)	Co-polymerization of sulfonic acid group suitable for latex nano-particle agglomeration.	RT	2 h	60 nm Latex	[48]
Poly(BuMA-*co*-AMPS-*co*-EDMA)	Co-polymerization of sulfonic acid group suitable for latex nano-particle agglomeration.	RT	2 h	65 nm Latex	[49]
Poly(BuMA-*co*-AMPS-*co*-EDMA)	Co-polymerization of sulfonic acid group suitable for latex nano-particle agglomeration.	RT	2 h	65 nm Latex	[50]
Poly(GMA-*co*-EDMA)	Introduction of surface cysteamine and subsequent cleavage of the disulphide bond, to reveal surface thiols. AuNP modification.	RT	N/A	10 nm Au	[127]
Poly(GMA-co-EDMA)	Generation of quaternary ammonium for IONP immobilization.	RT	N/A	19 nm iron oxide	[136]
Poly(EDMA)	Preparation of AuNP modified extraction pipette tip, for glycoprotein selectivity.	RT	N/A	20 nm Au	[146]
Poly(GMA-*co*-EDMA)	Coupling of α-glucosidase to AuNP modified monolith for PMME.	RT	3 h	15 nm Au	[147]
Poly(GMA-*co*-EDMA)	For AuNP immobilization, and functionalizing groups such as 3-mercapto propionic acid (CEC), 1-octadecanethiol (RPLC), and sodium 2-mercaptoethane sulfonate (SCX).	RT	N/A	15 nm Au	[148]

Table VI – Continued

Monolith	Reaction	Temperature °C	Time	Nano-particle type	Ref.
Poly(NAS-*co*-EDMA)	Surface generation of alkyne group, followed by thiol-yne photo-addition of cysteamine for AuNP modification.	RT	N/A	20 nm Au	[149]
Poly(GMA-*co*-EDMA)	Introduction of surface AuNPs by generation of surface thiols from surface epoxide groups. Two methods were used: (i) hydrogen sulfide, and (ii) cysteamine.	100 –RT RT	30 min 2 h 20 min	40-50 nm Au	[150]
Poly(BuMA-*co*-EDMA)	Preparation of thiolated and aminated surfaces and comparison of immobilization pathways for AuNPs.	RT	N/A	20 nm Au	[151]
Poly(GMA-*co*-EDMA)	Immobilization of SNPs upon a polymer monolith.	RT	60 min	50 nm Ag	[144]
Poly(HEMA-*co*-EDMA)	Generation of polymer monolith extraction device within a pipette tip, immobilized with IONPs via surface grafted functional groups (META).	RT	N/A	20 nm iron oxide	[152]
Poly(NIPAAm-*co*-GMA-*co*-EDMA)	Generation of γ-alumina nano-particle modified monolith for PMME.	60	14 h	10-20 nm Al_2O_3	[153]
Poly(GMA-*co*-EDMA), poly(BuMA-*co*-EDMA), poly(GMA-*co*-BuMA-*co*-EDMA)	Immobilization of Pd/Pt NFs *via* aminated surface groups.	RT	72 h	Pd/Pt NFs	[154]
Poly(GMA-co-EDMA)	Coating of monolith surface with graphene and graphene oxide nano-sheets for PMME.	RT	N/A	Graphene nano-sheets	[155]
Poly(GMA-*co*-EDMA)	Encapsulation of MWCNTs and surface attachment of MWCNTs *via* surface aminated groups.	RT	N/A	MWCNTs	[156]

MWCNTs), graphene, and nano-diamonds [27-29]. Chambers *et al*. described both the encapsulation method and the surface modification of polymeric monolithic columns with MWCNTs [155]. They prepared poly(GMA-*co*-EDMA) monolithic columns, which were treated with ammonium hydroxide, resulting in an aminated surface. To reduce the length of the MWCNTs, they were subjected to a cutting procedure, which resulted in the generation of carboxylic acids at the end of the tube walls. This rendered the tubes slightly hydrophilic; however, sufficient hydrophobicity was maintained. The unmodified generic column did not support

the separation of any alkylbenzene analytes (benzene, toluene, ethylbenzene, propylbenzene, butylbenzene, and amylbenzene). However, by following modification, all seven peaks in the sample mixture were resolved, with an efficiency of 23,000 N m^{-1} obtained for benzene. The separation was improved by reducing the average pore size (thus increasing the surface area); however, a three-fold increase in pressure drop across the column was also obtained. The group postulated that the increase in surface area also resulted in an increase in MWCNT coverage, thus accounting for the increased efficiency (40,000 N m^{-1} for benzene).

12 Concluding Remarks

To change the inherent functionality of a polymeric monolithic column, post-polymerization can be used, incorporating a plethora of possible reactions performed upon the polymeric substrate. Thus far, post-polymerization reactions have been shown to increase efficiency, increase stability of immobilized molecules (especially biomolecules), and to introduce a variety of surface functionalities with relative technical ease. Co-polymerization of a functional monomer can be used to produce the specific surface functionality; however, due to the incorporation of the reactive group within the polymer globules, it reduces the surface capacity, or the overall sites available for reaction at the monolith surface. To counter such a problem, the grafting of additional polymeric network can be used, resulting in the specific functionality required. Grafting by means of thermal or UV initiated polymerizations in the presence of an initiator and monomer, or living polymerization methods (whereby latent radicals initiate the addition reactions) are used routinely. However, polymer monoliths contain a large population of throughpores, thus reducing the overall surface area of the substrate. The addition of nano-architectures has been demonstrated to enhance the inherent surface area, and to facilitate the addition of ligands. The combination of these methods may enhance the control of meso- and micro-pores in future polymeric monolithic substrates as well as ligand density, particularly in the preparation of hypercross-linked monoliths, for which subsequent modifications are currently in their infancy [126]. The use of short polymerization times may also be used in conjunction with modification techniques such as thiol-ene clicking, as such short polymerization times produce columns with a surface rich in di-vinyl groups. Post-polymerization techniques can, therefore, offer a wide variety of surface functionalities, whilst maintaining separate control of the monolith morphology and selectivity.

Abbreviations:
2-acrylamido-2-methyl-1-propanesulphonic acid AMPS, α, α'-azobisisobutyro-

nitrile AIBN, adenosine triphosphate ATP, atom transfer radical polymerization ATRP, benzoyl peroxide BPO, bovine serum albumin BSA, butyl methacrylate BuMA, capillary electrochromatography CEC, capillary electrophoresis CE, cyclic olefin copolymer COC, concanavalin A Con A, 2,2-dimethoxy-2-phenylacetophenone DAP, dibutyl tin dilaurate DBTL, dichloroethane DCE, dimethylformamide DMF, divinylbenzene DVB, ethyleneglycol dimethacrylate EDMA, electroosmostic flow EOF, green fluorescent protein GFP, glycidyl methacrylate GMA, gold nano-particle(s) AuNP(s), hydroxyethyl methacrylate HEMA, hydrophilic interaction liquid chromatography HILIC, human serum albumin HSA, iminodiacetic acid IDA, isocyanoethyl methacrylate IEM, ion exchange IEX, immobilized metal affinity chromatography IMAC, iron oxide nano-particle(s) IONP(s), lauryl methacrylate LMA, methylenebisacrylamide MBA, *N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium betaine MEDSA, methacryloyloxyethyl trimethylammonium chloride META, methyl methacrylate MMA, 4-methyl styrene MST, multi-walled carbon nano-tube(s) MWCNT(s), *N*-acryloxysuccinimide NAS, poly(N-isopropylacrylamide) NIPAAm, nano-flower(s) NF(s), oligoethylene glycol OEG, polyethyleneglycol methacrylate PEGMA, polyethylimine PEI, polymer monolith micro-extraction PMME, Teflon PTFE, reversible addition-fragmentation chain transfer RAFT, reversed-phase RP, ring opening metathesis polymerization ROMP, room temperature RT, scanning capacitively coupled contactless conductivity detection sC^4D, strong cation exchange SCX, scanning electron microscope SEM, silver nano-particle(s) SNP(s), solid phase extraction SPE, sulfopropyl methacrylate SPM, styrene STY, single-walled carbon nano-tube(s) SWCNT(s), tris(2-carboxylethyl)phosphine hydrochloride TCEP, 2,2,6,6-tetramethylpiperidyl-1-oxyl TEMPO, trimethylolpropane trimethacrylate TRIM, vinyl azlactone VAL, vinylbenzyl chloride VBC, wheat germ agglutinin WGA.

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