Poly(2-oxazoline) Hydrogels: State-of-the-Art and Emerging Applications

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Abstract

The synthesis of poly(2-oxazoline)s has been known since the 1960s. In the last two decades they have risen in popularity thanks to improvements in their synthesis and the realization of their potential in the biomedical field due to their non-fouling or 'stealth' properties, stimuli responsiveness and tailorable properties. Even though the bulk of the research to date has been on linear forms of the polymer, they are also of interest for forming network structures due to the relatively easy introduction of reactive functional groups during synthesis that can be crosslinked under a variety of conditions. In this opinion article, we briefly review the history of poly(2-oxazoline)s and examine the *in vivo* data on soluble poly(2-oxazoline)s to date in an effort to predict how hydrogels may perform as implantable materials. This is followed by an overview of the most recent hydrogel synthesis methods, emerging applications and concludes with a section on the future directions predicted for this fascinating and versatile class of polymer.

1. Introduction

Synthetic hydrogels have become an invaluable tool to a number of emerging biomaterial applications requiring high water content gels. These include drug delivery, tissue engineering and subclasses of these, such as 3D cell culture, and more recently, biofabrication. Concomitant to the advances of these fields, there has been an increase in the variety of crosslinking chemistry and sophistication contained within the underlying hydrogels since the first synthetic hydrogel, poly(2-hydroxyethyl methacrylate), was described by Wichterle and Lim in 1960.[1] Despite almost 60 years' worth of research into

synthetic hydrogels, the majority of products translated to market can be classified into just a few categories, namely contact lenses, hygiene products and wound dressings. Undoubtedly more hydrogels will enter the market in the foreseeable future in the form of drug delivery and tissue engineering devices as the technologies mature.[2]

For many of the established bulk hydrogel applications, e.g. hygiene products and contact lenses, the polymerization mechanism has been around low-cost traditional free-radical polymerizations. More recently though, the focus has been around developing and exploiting low energy crosslinking chemistry (light activated,[3] 'click' reactions,[4] enzyme mediated,[5] thermogelation[6]) of polymeric precursors so that biological components can be included (e.g. peptides,[7] growth factors,[8] cells[9]). Furthermore, the use of controlled and living polymerization methods for the preparation of the hydrogel precursors provides control over the spacing of crosslinks leading to near-ideal network structures.[10] A key development towards advanced hydrogels was the work of Hubbell and Lutolf who prepared polyethylene glycol (PEG) hydrogels based on enzymatic crosslinking with cell-instructive degradation points by using cleavable peptide substrates as crosslinkers.[5] This evolution of the hydrogel from a passive material into an active material with tissue-like characteristics[11-15] has reinvigorated the field.

One of the most successful and intensively studied synthetic polymers for the preparation of hydrogels is PEG. Soluble PEG (i.e. non-crosslinked) has been ubiquitous in biology laboratories for precipitation of biomolecules and cell fusion since the mid-1950s.[16] In his commentary, 'The Origin of pegnology', Frank Davis describes the beginnings of his pioneering work into PEGylation of proteins in the late 1960s for reducing immunogenicity following intravenous injection of liposomes and therapeutic proteins. What set PEG apart from other polymers of the time was that it was available as well-defined monomethoxy PEG with a single hydroxyl-functionalized terminus, prepared by living anionic polymerization. This long history of using PEG in biological applications together with the availability of well-defined structures (now including star-architecture PEGs) and its well-known 'stealth'/ non-fouling behaviour have made it a popular choice as a hydrogel material for biomedical applications.

In recent years, poly(2-alkyl/aryl-2-oxazoline)s (PAOx) (Figure 1) are increasingly being proposed as an alternative to PEG.[17-19] Much of the focus on PAOx has been on the ease of synthesis as well as on self-assembling non-crosslinked materials. Nonetheless, there is also interest in using PAOx for hydrogels based on the additional functionality available through the side-chains allowing for high degrees of conjugation or crosslinking. Perhaps the earliest example of a PAOx hydrogel was from Litt who presented at a 2012 symposium on PAOx his early work in the 1960s on using thiol-ene photochemistry to crosslink functional PAOx,[20] although there is no peer reviewed account of this work. Later in the 1980s and early 1990s, Chujo and Saegusa published a large body of innovative

work on PAOx hydrogels based on covalent, dynamic covalent and supramolecular crosslinking.[21-32] These reports appeared, however, before the field of biomaterials became widespread and so their materials were not used with biological systems.

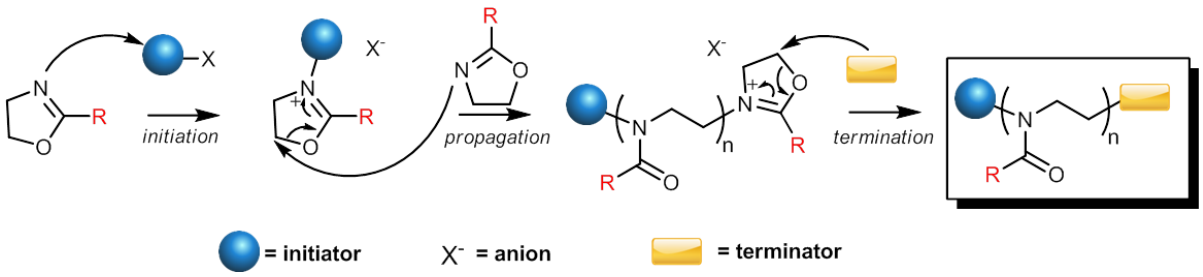


Figure 1: General scheme for the cationic ring opening polymerization of 2-oxazolines and the structure of the resulting poly(2-alkyl/aryl-2-oxazoline)s.

To understand the evolution of PAOx as a hydrogel material it is useful to look at the timelines in which it has been developed. PAOx was first synthesized experimentally in the 1960s by four independent groups[33-36] and later on industrial scale in the 1980s.[37] PAOx is pseudo-peptidic in structure (the amide is on the side-chain, not the backbone as with peptides) that can be prepared with a variety of 2-substituted-2-oxazoline monomers leading to a range of physical properties such that it can be considered a class of polymers.[38] The cationic ring opening polymerization (CROP) of 2-alkyl/aryl-2-oxazoline monomers is used to form the polymer but the process is susceptible to chain transfer and other modes of uncontrolled termination, especially when aiming at higher molar mass polymers and in presence of nucleophilic impurities, such as water. This meant that for a long time it was not thought of as a polymer for which low polydispersity could be achieved for higher molar mass polymers. We can speculate that Frank Davis would have been aware of PAOx during his library searches, but disregarded it since at the time PAOx was only available as an ill-defined polymer. Thus, PAOx remained as a curiosity to just a few research groups struggling to gain more widespread attention, until recently.

The renewed interest in PAOx can be traced to the development of methods for accelerating reaction rates for the CROP of 2-oxazolines using microwave assisted polymerization in 2004.[39] Until this time the synthesis of PAOx was slow, often taking > 6 hrs up to weeks, whereas similar results could be achieved using microwave heating in under 1 min. This enabled the relatively easy preparation of libraries of PAOx homopolymers and copolymers taking advantage of the variety in structures possible. As an aside, it is ironic that the recent discovery that low dispersity, high molar mass PAOx is feasible under meticulously dry conditions uses conventional lower temperature conditions and not microwave heating.[40]

Technically, microwave heating made the synthesis of PAOx much more accessible, but its use as a biomaterial was strongly influenced by the discovery of its non-fouling properties by the group of

Textor.[41] By synthesizing poly(2-methyl-2-oxazoline) (PMeOx) grafted onto poly(L-lysine) they were able to attach the polymer as a mono-layer to negatively charged surfaces to show that PMeOx had slightly better anti-fouling properties compared to PEG. This was contrary to earlier findings by the group of Hubbell[42] who found that polyethylene terephthalate (PET) surfaces coated with high molar mass poly(2-ethyl-2-oxazoline) (PEtOx) had higher protein adsorption, and hence cell attachment, compared with polyethylene oxide (PEO). However, they compared only one molar mass of PEtOx with multiple molar masses of PEO and the exact composition and homogeneity of the PEtOx surface was not fully elucidated with some speculation that it changed over time. Especially interesting is the report by the group of Textor in which it was shown in a direct comparison that PAOx coated surfaces were more stable under oxidative conditions compared to PEG, ascribed to the side chain degradation mechanism of PAOx versus the main-chain degradation of PEG.[43]

Two very recent developments that will potentially ensure the continued growth in interest in PAOx are the synthesis of high molar mass, low dispersity polymers using meticulously dry conditions[40] mentioned above, and the 'first in human' clinical trials[44] of a PAOx-drug conjugate. These two developments, reported by two different research teams, may be considered important developments needed for widespread use of PAOx in the biomedical field.

This mini-review will provide an overview of very recent developments in hydrogels based on PAOx and aims to address the potential advantages of using PAOx hydrogels over other more established materials, including PEG, in the context of biomedical applications. It is not intended to be an exhaustive review of all work on PAOx hydrogels for which the reader is directed to excellent reviews by Adams and Schubert[18] in 2007, Kelly and Wiesbrock[45] in 2012, and Hartlieb *et al.*[46] and Zahoranová and Kronek,[47] both in 2015.

2. Biocompatibility and Excretion of PAOx

Almost without exception, all the recent literature on PAOx hydrogels is targeted towards biomaterial applications, and while there are many *in vitro* studies with cell lines, or in some instances, primary cells, *in vivo* studies on implanted PAOx hydrogels are non-existent to the best of our knowledge. It is true that *in vitro* data is sufficient for PAOx-based sensor applications (discussed later),[48-50] but for any drug delivery, implant or tissue engineering application, *in vivo* data is critical to understand the degradation, circulation kinetics, excretion and immune responses.

In the absence of these data we can speculate that PAOx hydrogels implanted into animals as drug/cell delivery devices or tissue engineering scaffolds will have some level of foreign body host response similar to other non-ionic synthetic hydrogels. For example, photo-crosslinked PEG is known to recruit macrophages when implanted subcutaneously in mice[51] as a result of non-specific protein adsorption, despite being widely considered as a low protein-fouling polymer. Given the similar physical properties of PAOx to PEG and shared low-fouling attributes,[41] a similar

inflammatory response may be anticipated if implanted *in vivo*. Of course, any chemical modification will influence the response, as will the animal species and site of implantation. What is important, though, is to design degradable hydrogels and to evaluate the fate of soluble degradation products (which can be predicted if degradable crosslinks are used) and non-crosslinked leachable polymer chains. Other oxidative degradation products may be expected as predicted by accelerated studies using reactive oxygen species.[52] Thus, in the near absence of literature on *in vivo* studies of PAOx hydrogels, it is worthwhile to discuss the animal studies on soluble PAOx as a proxy for how leachable and degradation products from hydrogels may be tolerated *in vivo*.

A summary of all the *in vivo* studies on PAOx over the last 40 years are listed chronologically in Table 1. The first entry – the inclusion of PEtOx as an approved adhesive in food packaging, is an exception as it does not describe animal work but is relevant as it is the first formal acknowledgment that the polymer may be non-toxic. Of the studies from 1989 to 2017 it is interesting to note that half of them were reported in just the last three years, highlighting the maturation of PAOx technology for biomaterials.

The studies listed in Table 1 can be crudely categorized into: 1) biodistribution of soluble polymers, 2) biodistribution of liposomes or micelles (with and without loaded drugs), 3) toxicity and efficacy of polymer-drug conjugates, and 4) a hemostatic dressing.

Table 1. Summary of *in vivo* studies on PAOx over the last 40 years.

PAOx System	Year	Experiment	Key Results	Reference
PEtOx	1977	-	PEtOx listed as FDA approved adhesive in packaging for food	Food and Drug Administration[53]
PMeOx-co-2-(4-hydroxyphenyl)-2-oxazoline	1989	Biodistribution ^{125}I labelled copolymers injected iv in mice	Blood clearance measured after 24 hrs, accumulation in skin and muscle but not in organs of the mononuclear phagocyte system	Goddard *et al.*[54]
Lipids prepared from PMeOx or PEtOx (1.5-5 kDa) conjugated to distearoylphosphatidylethanolamine	1994	^{67}Ga-labelled liposomes injected iv into rats	Long circulation times, protection from rapid recognition	Woodle *et al.*[55]
Lipids prepared from PMeOx or PEtOx (1.5-5 kDa) conjugated to distearoylphosphatidylethanolamine	1996	^{125}I loaded liposomes (polymer-lipid was 5 mol % of phospholipid content) iv injection in mice	Relatively long blood circulation times	Zalipsky *et al.*[56]
Radiolabelled PEtOx, PMeOx via end group chelator.	2007	^{111}In labelled biodistribution experiment in mice following iv injection	Rapid clearance from the blood, predominately excreted via kidneys; slightly higher for PMeOx compared with PEtOx suggesting possible binding to plasma proteins by the latter. Negligible tissue accumulation.	Gaertner, Luxenhofer *et al.*[57]

P(MeOx-b-ButOx-b-MeOx) copolymer	2010	Paclitaxel loaded micelles injected into Lewis Lung carcinoma tumour bearing mice	Significant tumour burden after first injection of Paclitaxel loaded P(MeOx-b-ButOx-b-MeOx) at a dose of 10 mg/kg.	Luxenhofer *et al.*[58]
PMeOx, PEtOx, PPropylOx	2011	Insulin conjugates iv to rats; BSA conjugates into rabbits and tested for antibodies; toxicity based on acute and chronic dosing in rats	Sustained lowering of blood glucose in rats; attenuation of BSA immunogenicity; high maximum tolerated doses in rats	Viegas *et al.*[59]
PEtOx-polylactic acid-polyethyleneimine micelles	2014	Delivery of mini-circle DNA and doxorubicin to a breast cancer mouse model	Good gene expression and retention of the micelles within the tumour cite with little gene expression in the spleen, kidneys, liver and lung.	Gaspar *et al.* [60]
PMeOx conjugated liposomes	2015	Injected into rats and mice and circulation times measured.	Rapid clearance after second injection due to possible IgM response	Kierstead *et al.*[61]
^{89}Zr labelled PEtOx-desferrioxamine chelator	2016	Micro Positron Emission Tomography of mice injected iv and *ex vivo* biodistribution	Rapid clearance for 20, 40 kDa, higher molecular weight leads to longer blood circulation times, some tissue accumulation above 70 kDa	Wyffels *et al.* [62]
^{89}Zr and ^{18}F labelled PEtOx	2017	Micro Positron Emission Tomography of mice injected iv	Rapid clearance of 5 kDa polymers	Glassner *et al.*[63]
PEtOx-rotigotine conjugates	2017	Multiple small animal studies, monkeys, humans	Sustained levels of free rotigotin in plasma of monkeys following cleavage from PEtOx backbone; improved 'on time' in MPTP model monkeys; improved rotational behavior in 6-OHDA lesioned rats; high clearance rates via kidneys; safety demonstrated in first-in-man phase I clinical trial	Moreadith *et al.*[44]
PAOx-NHS coated on a collagen sponge	2017	Heparinised pigs	Hemostasis achieved	Boerman *et al.*[64]
^{125}I labelled PEtOx-doxorubicin conjugates	2017	Female C57BL/6 mice, biodistribution and therapeutic activity	Gradual blood clearance with some accumulation in the tumor, spleen and liver. Reduction in volume of tumor.	Sedlacek *et al.*[65]

The hemostatic dressing listed in the Table above is the only animal study of a material resembling a PAOx hydrogel to date. In their study, the group of Van Hest[64] together with tissue-sealant company GATT Technologies, used *N*-hydroxysuccinimide (NHS) ester functional copolymer of 2-*n*-propyl-2-oxazoline (PropOx) and 2-methoxycarbonylethyl-2-oxazoline (MestOx[66]) coated onto a collagen sponge. Even though this is not a PAOx hydrogel, the excess NHS ester groups crosslinked with blood and ECM proteins to *in situ* form a hydrogel coating that induces blood clotting and stops bleeding (Figure 2). A test of the most promising composition with the shortest gelation time (<1 min)

was used to test the suitability of the dressing as a hemostatic agent towards bleeding in heparinized pigs. Other than an assessment of the bleeding no adverse effects of the polymer were reported.

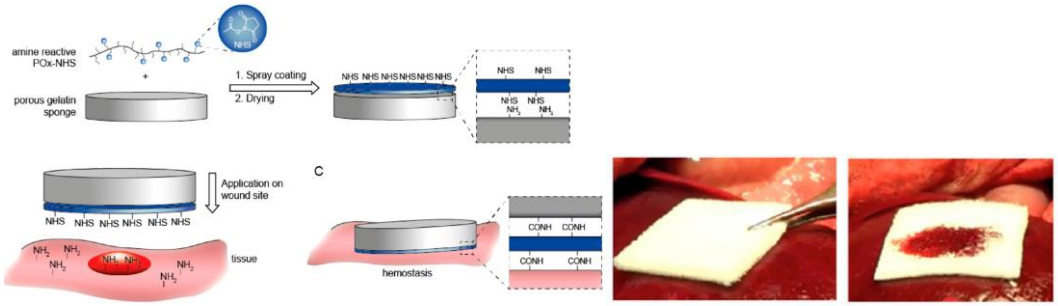


Figure 2: Left: Schematic showing preparation of a PAOx-NHS coating on a gelatin sponge as a hemostatic dressing; Right: the dressing on a pig spleen at 0 and 5 minutes. Adapted from Boerman *et al.*[64]

Of the studies examining liposomes, long circulation times are reported in all cases with the exception of Szoka and co-workers[61] who observed accelerated blood clearance after a second injection of PMeOx conjugated liposomes into Male Wistar rats. Even though the half-life of 30.5 ± 1.3 hours after the first dose was one of the highest for the polymers studied, the half-life dropped to only 1.9 ± 0.5 hours after the second dose. They postulate that this is due to the generation of anti-PMeOx-IgM, similar to the anti-PEG-IgM found in 25% of healthy blood donors. More general, they found a correlation between high polymer intrinsic viscosity and good circulating times after the first administration and accelerated clearance after the second administration. The explanation for this observation is still under investigation and it is unclear whether the use of rather ill-defined PMeOx (dispersity around 1.4) is related to the observed effect.

Unlike liposomes, soluble PAOx is rapidly cleared from the blood and shows little tissue accumulation below approximately 70 kDa.[62] The first study of biodistribution by Goddard *et al.* reported in 1989 using ^{125}I labelled PMeOx-*co*-2(4-hydroxyphenyl)-2-oxazoline found some accumulation of the tracer in the skin and muscle.[54] A similar study on PMeOx and PEtOx by Gaertner, Luxenhofer *et al.*[57] found negligible tissue accumulation and postulate that the earlier Goddard *et al.* study lacked the control over polydispersity meaning the tissue accumulation could have been due to high molecular weight fraction.

In summary it appears there is overwhelming evidence that soluble PAOx is well-tolerated and does not accumulate in the body. Therefore, it should be possible to design implantable or injectable PAOx hydrogel devices of which the leachable or degradable products are expected to cause no harm. What is less known is how the body will react to the hydrogel itself? If PEG hydrogels are a guide, then

caution needs to be employed. For instance, one of the largest studies involving implanted PEG hydrogels was in the 1990s investigating PEG hydrogel coatings on pancreatic islets with the aim of reducing the need for immunosuppression for type I diabetes recipients. While promising results were observed in rodents, the study failed in non-human primates due to aggressive reaction to the coatings.[67] This highlights the dangers in extrapolating from small animals to large animals, or indeed, from *in vitro* to any animal study, yet at the same time can help guide experimental design used with PAOx hydrogels in biomedical applications.

3. Recent Developments in PAOx Hydrogel Synthesis

One of the most prolific periods of research on innovative crosslinking chemistry for PAOx was in the late 1980s and early 1990s single-handedly by the group of Chujo and Saegusa who published at least a dozen articles[21-32] revealing new ways of forming crosslinked networks based on PAOx. Their work has been reviewed before (e.g. in the review articles mentioned earlier) and so will not be discussed here other than to mention that their work was almost exclusively focussed on the synthesis of the polymers and crosslinking chemistry and the stability of the gels without investigation of potential applications (at least in the open literature). Since the work by Chujo and Saegusa, the number of new functional PAOx has increased significantly[68] and, hence, the variety in crosslinking chemistry has expanded too. The discussion here will be confined to very recent developments; note that some of the references used here will appear again in the later section on applications where appropriate.

3.1 Crosslinking During Polymerization

The formation of a continuous network during polymerization of 2-alkyl-2-oxazoline monomers via the inclusion of a bis-2-oxazoline is a scale-able, convenient and rapid method for creating hydrogels not readily accessible with other non-ionic hydrogels (e.g. PEG, PVA). The disadvantages, however, are that the networks are non-degradable, and because the reaction mixture must be heated to high temperatures and performed in absence of water, the swelling in water and any incorporation of biologics can only occur after network formation and solvent removal.

In a demonstration of the versatility of the polymerization-gelation method, Kronek and co-workers synthesized a series of hydrogels from EtOx and a homologous series of three bis(2-oxazoline) crosslinkers, with butyl, hexyl or octyl spacers between the 2-oxazoline rings and methyl-4-nitrobenzenesulfonate as an initiator with a polymerization temperature of 110 °C for 5 hrs.[69] As expected, an inverse relationship between the swelling of the hydrogels in water and the length of the alkyl spacers in the bis(2-oxazoline) crosslinkers was observed. The moduli of the hydrogels increased with increasing crosslinker content and spacer length, attributed to a higher proportion of inter-chain crosslinking for longer spacers. Toxicity experiments of extracts with 3T3 fibroblasts

revealed some toxicity attributed to residual monomer and benzonitrile (their polymerization solvent) while growing cells on the hydrogels was most successful for the soft hydrogels. It should be noted that a number of factors, including leached compounds, substrate stiffness and protein adsorption from the media, will all play a role. Some insight into the intended application of the hydrogels was evident from their experiments involving freeze drying of the hydrogels to introduce porosity, similar to Hickey and co-workers,[70] followed by seeding of pancreatic β cells inside the pores which formed aggregates and remained viable for 12 days.

Schubert and co-workers also used a bis-functional 2-oxazoline to form networks with EtOx, but with a phenyl group as the spacer.[71] Their new phenyl-1,4-bis-oxazoline is a structural isomer of the previously reported crosslinker phenyl-1,3-bis-oxazoline reported by Wiesbrock and co-workers[72] and was used for the first time to form a gel during CROP of EtOx in a one-pot, microwave assisted reaction. The highest gel fraction reported was 84% and solid and swollen state NMR spectroscopy identified a small amount of crosslinking incorporated through just one of the bis-oxazoline rings. The hydrogels were investigated for stabilization of coagulation factor VIII as a more convenient storage method rather than preparing from dry powder immediately before injections. Their hypothesis is that the pseudo-peptidic structure of PAOx may lead to a high affinity, and hence improved stabilization, between the polymer and protein. Their test method consisted of loading multiple hydrogels into well plates, washing and addition of factor VIII, incubation and then centrifugation and measurement of residual factor VIII activity. It was observed that the crosslinking density was highly influential towards protein stability yet stability at 7 days was significantly improved compared with any of the controls.

3.2 Crosslinking of Polymeric Precursors

The use of polymeric precursors to create PAOx hydrogels is more complex than the methods described above, but it is an attractive alternative as it allows for potentially better control over the network structure and may be used to reduce toxicity during the crosslinking process due to the low energy input required to go from soluble functional polymers to an insoluble network. Furthermore, the issues associated with residual monomers or solvent leaching post-gelation can be avoided. That is not to say that all polymeric precursor approaches use non-toxic crosslinking conditions and there are many examples of inclusion of organic solvents,[29, 73] toxic reagents,[22, 25] heat,[23] and high energy radiation[74] being used in crosslinking. If, however, water soluble macromonomers are used with mild crosslinking reactions, then it is possible to form gels under non-toxic conditions. Most of the studies to date include only proof-of-principle cell toxicity studies and no long-term or *in vivo* data exists yet other than that of Van Hest and co-workers[64] discussed in Section 2.

Many of the functional 2-oxazoline monomers are hydrophobic, however, copolymerizing with hydrophilic monomers can lead to water-soluble polymeric precursors. For example, copolymerization of 2-undecenyl-2-oxazoline (DecenOx) with EtOx results in statistical copolymers soluble in ethanol[73] that form micelles in aqueous conditions.[75] Conversely, when DecenOx is copolymerized with the more hydrophilic MeOx, the product with DecenOx content up to 5% is water soluble.[7, 76, 77] The ability to retain water solubility even when using hydrophobic monomers such as DecenOx is a function of the high hydrophilicity of PMeOx. The striking degree of hydrophilicity of PMeOx has been demonstrated using HPLC to show even greater affinity for water compared with a column stationary phase than PEG.[59] Nonetheless, in our recent work[76] we found that there is a certain degree of hydrophobic association between the hydrophobic DecenOx groups resulting in highly efficient photo-gelation consisting of competition between the thiol-ene radical addition reaction and vinyl homocoupling. The same phenomenon was not observed with PMeOx-ButenOx copolymers.

The use of aqueous conditions for crosslinking is not a prerequisite if biologics are not being used and organic solvents can be utilized to increase the range of crosslinkers used. Kronek's group[78] used dithiothreitol, 1,3-propanedithiol, 1,6-hexanedithiol and 1,9-nonanedithiol to crosslink copolymers of PMeOx-ButenOx or PEtOx-ButenOx (each in a comonomer ratio of 90:10) in 20% ethanolic solutions using 365 nm light irradiation. Following gelation, the ethanol was exchanged to water and toxicity determined on extracts and in direct contact with 3T3 fibroblasts. The extracts were found to be toxic in high concentrations but in all cases direct contact toxicity was low, and similarly to the previous work by the same group,[69] the more hydrophobic the crosslinker the lower the swelling. he softness of the hydrogels was also measured using a small-probe indentation method revealing relatively low moduli in between 6.3 kPa and 128 kPa for the most and least swollen hydrogels, respectively.

The group of Luxenhofer has also used MeOx as a monomer to prepare water-soluble PAOx to encapsulate cells for biofabrication[79] (discussed more in section 4.2.3). Rather than using a chemically crosslinking system, they found that CROP of 2-*n*-propyl-2-oxazine using methyl triflate (MeOTf) as the initiator followed by addition of a PMeOx block to the living system resulted in polymers that would thermally gel around 20-40 °C, depending on composition. The use of 2-oxazines in combination with 2-oxazolines is a novel way of extending the range of possibilities of this class of polymers also recently highlighted by Kempe.[80]

The use of telechelic PAOx as the crosslinker is another method for making networks and takes advantage of the facile and, arguably under-utilized, end-group functionalization available during the termination step of the CROP of 2-oxazolines. The group of Tiller recently expanded on their work into PAOx based conetworks comprising of nanostructured interconnected polymer phases by

polymerizing di-methacrylamide terminated PMeOx with butyl acrylate in 1-methoxy-2-propanol as the solvent. The macromolecular crosslinker was prepared by using a difunctional initiator, *p*-dibromoxylene, followed by termination of the polymerization with DMAP-methylacrylamide to achieve 95% functionalization.[81] These conetworks were used to activate lipase from *C. antarctica* for application in organic solvent enzymatic catalysis.

The telechelic PAOx approach has also been used by the group of Jordan who synthesized di-methacrylate terminated PMeOx (using methacrylic acid to terminate) and mixed methacrylate-alkyne PMeOx (using propargyl *p*-toluenesulfonate as the initiator and methacrylic acid to terminate the reaction).[82] The α-alkyne-ω-methacrylate was subsequently used in a Huisgen cycloaddition 'click' reaction to incorporate a disulphide core to the crosslinker. The microbeads synthesized from these telechelic PMeOx crosslinkers are described more in Section 4.2.1.

3.3 Irradiation

Ionizing radiation is an attractive method for modifying polymers and has a long history in polymer processing, especially in creating a variety of crosslinked materials from hydrogels[83] to fluoropolymers.[84] Radiation processing of PAOx, however, has received very little attention in the literature. The group of Voit created nanogels using 20 kGy dose of electron beam irradiation of poly(*N*-isopropylacrylamide) (PNiPAAm) grafted with poly(2-carboxyethyl-2-oxazoline)[85] or PEtOx,[86] whereby crosslinking was assumed to occur through the dense hydrophobic PNiPAAm core with no mention of the radiation chemistry of the PAOx component.

Around the same time as the reports by Voit, Ali and AlArifi reported hydrogels prepared from mixtures of PEtOx and acrylic acid (no solvent was mentioned so we must assume the acrylic acid was the solvent for PEtOx) that were irradiated up to 40 kGy. However, when the PEtOx content was increased no gel was observed leading them to conclude that PEtOx is not a radiation cross-linkable polymer.[87] Very recently Sedlacek *et al.*[88] found the opposite. They examined the potential for electron beam and gamma irradiation to be used in sterilization of PAOx and found that for dilute solutions (1-5 wt. %) of PEtOx in water, hydrogels were formed in yields of 60-85% with >5 kGy for electron beam and >50 kGy for gamma irradiation. A mechanism for crosslinking is proposed via a radical formed at the methylene on the ethyl side chain as a result of C-H dissociation, presumably to form H-type crosslinks following combination with the same species of radical on an adjacent chain. The authors mention on-going work exploring this somewhat serendipitous result.

It appears that the radiation conditions are highly determinant as to whether a network is obtained or not. The solvent, concentration, polymer molar mass and radiation dose are all important. Sedlacek *et al.*[88] also report the irradiation of solid PEtOx and show chain scission. This is consistent with our work on irradiating solid PEtOx and PEtOx plasticized with a small percentage of water which

showed predominantly chain scission by size exclusion chromatography when exposed to gamma radiation up to 100 kGy (unpublished data).

In summary, the variety and sophistication of the crosslinking methods for PAOx continues to grow taking advantage of the possibilities of incorporating functionality in the initiating group residues, terminating agents, backbone and side-chains. Other methods, such as irradiation, offer a relatively cheap and highly scalable method for producing PAOx hydrogels.

4. Emerging Applications

4.1 Drug Delivery

PAOx has been used as a drug delivery carrier in the form of nanoparticles,[58, 89] soluble polymer-drug conjugates[44, 59] and eroding systems,[90, 91] but very few studies have been reported using PAOx hydrogels or other networks for drug delivery. Considering the possibilities of making PAOx gels with custom polarity and swelling, these could be viewed as attractive drug delivery systems compatible with a range of drugs and tailored release profiles.

The group of Wiesbrock[92] have demonstrated this approach by making libraries of hydrogels incorporating ethyl, butenyl, nonyl, and decenyl side-chains that were used to encapsulate active pharmaceutical ingredients (APIs). They used two routes to synthesize the hydrogels – either using bifunctional monomers prepared via thiol-ene photochemistry of alkene functional 2-oxazoline monomers with dithiols including an ester as degradable linker followed by microwave assisted polymerization, or first the formation of polymers followed by thiol-ene photo-crosslinking. The post-polymerization crosslinking method allowed for incorporation of ibuprofen or eosin B. The latter was used for a release study with a terpolymer of PEtOx-NonOx-ButenOx revealing that at higher pH more eosin was released. Interestingly, there was no burst release and the increase in eosin in solution over time was linked to the hydrogel degradation via hydrolysis of the ester containing crosslinker; this may suggest that the eosin was physically bound to the polymer which supports our findings that the use of eosin will stain PAOx hydrogels.[70] The work by the group of Wiesbrock follows on their previous study imbibing and releasing small molecules to and from hydrogels prepared with a wide range of 2-oxazoline monomers varying in hydrophilicity.[72]

Not only are PAOx hydrogels attractive for delivery of small molecules, but they have also been used with silver ions for antimicrobial coatings. The group of Demirel[93] used different molar mass PEtOx hydrogen bonded with tannic acid in a layer-by-layer approach to encapsulate silver nanoparticles. Films up to 80 nm thick were achieved using the dip coating method, which degraded at high pH, releasing the silver. Although the films are proposed as antimicrobial coatings, no bacterial assays were presented in that study.

Drug delivery using nanogels for cellular uptake is a method for delivery payloads directly to cells. Schubert and co-workers synthesized block copolymers of EtOx and Boc-protected 4-amino-butyl-2-oxazoline, followed by cleavage of the Boc group to create the free amine (Figure 3).[94] Subsequent preparation of self-assembled nanoparticles was performed by glutaraldehyde crosslinking via the free amine groups and titration of residual aldehyde groups with 6-aminofluorescein. By varying the amount of glutaraldehyde, the zeta potential of the nanogels could be modified based on the amount of free amine groups as a way of modulating cellular uptake. As their ultimate application is cell targeting following injection into the bloodstream, long circulation times are desirable, similar to the PAOx lipid studies summarized earlier in Table 1. Although this group did not perform circulation studies they did investigate their nanogels for induced hemolysis and erythrocyte aggregation and found no significant interaction of the nanogels with blood. Other recent studies on PEtOx modified nanoparticles[95] and PMeOx coated viruses,[96] each without the ability to vary the zeta potential, similarly found reduced interaction of PAOx modified materials with cells.

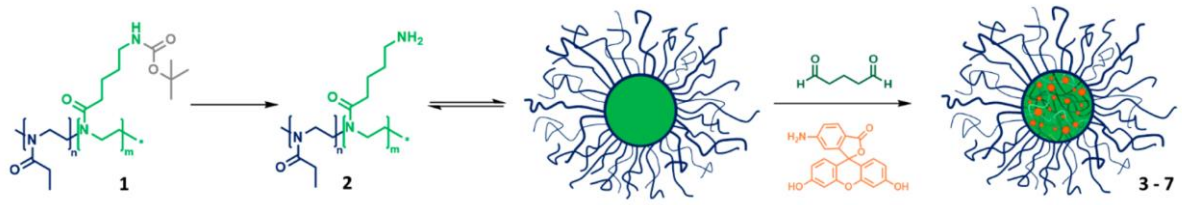


Figure 3: Structures of PEtOx-b-4-amino-butyl-2-oxazoline before and after deprotection of the Boc groups and schematic of the micellular encapsulation of 6-aminofluorescein. Taken from Hartlieb *et al.* [94]

4.2 Cellular Constructs

4.2.1 Cell Therapies

Cell therapies have long been hyped as a panacea for numerous disease states, with stem cells receiving the highest of expectations. Some of the promise of cell therapies has recently been realised, not from stem cells but from gene-modification of T cells. Very recently a new gene-modified immunotherapy, chimeric antigen receptor T-cell therapy (CAR-T), was approved by the Food and Drug Administration to treat leukemia and is expected to be followed by many other similar products. The role that hydrogels play in cell therapies will be minor, but nonetheless important. For instance, hydrogels can aid in the expansion of cells or carriers for transplantation.

The good tolerability of soluble PAOx in the body (Table 1) and the ability to create degradable hydrogels make PAOx hydrogels an interesting and emerging candidate material for use in cell therapies. A recent study by Jordan's group[97] used the surface of PAOx beads for neuronal cell culture ultimately intended for transplantation. Their beads were synthesized using an emulsion

polymerization of HEMA or 2-methacryloxyethyltrimethylammonium (METAC) with a $PMeOx_{30}$ dimethacrylate crosslinker prepared from the difunctional initiator, *trans*-1,4-dibromo-but-2-ene (Figure 4). The size of the beads from the emulsion process was quite disperse but could be selectively sieved to a range of 30-250 μm. The PAOx itself was passive whereas the positive charge of METAC ensured cell adhesion. Neither rat primary hippocampal neuronal cells nor the more adhesive HEK cells adhered to HEMA-PMeOx spheres and attempts to coat with poly(L-lysine) (PLL) via LbL proved problematic, but replacement of the HEMA with METAC improved HEK adhesion but not neuronal cells unless the PLL was also included.

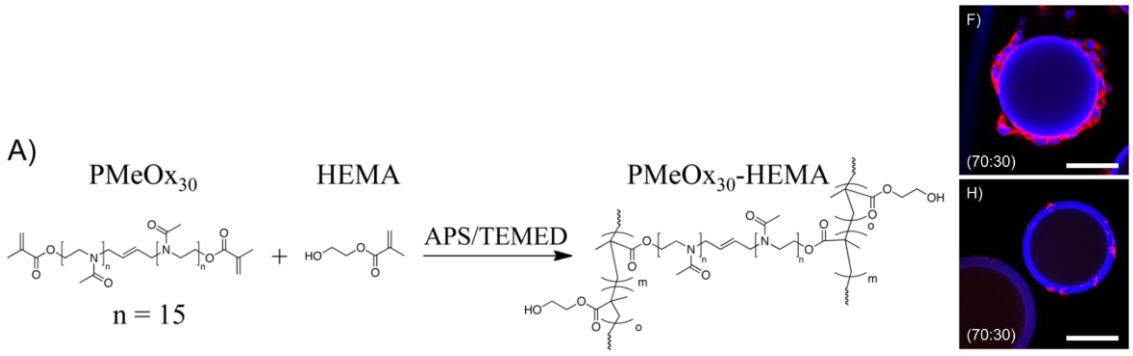


Figure 4: Precursors and structure of PMeOx-HEMA networks based on PMeOx-dimethacrylate synthesized from the difunctional initiator, *trans*-1,4-dibromo-but-2-ene and laser scanning confocal microscopy images of fluorescently stained HEK and neuronal cells on microgel particles. Modified from Platen *et al.*[97]

The same group later reported a continuous microfluidic method to produce uniform microbeads with a tailorable diameter of 50 to 500 μm without the need for sieving as an improvement over the emulsion batch process.[82] They used a short telechelic dimethacrylate $PMeOx_{30}$ crosslinker and a degradable disulphide PMeOx crosslinker (discussed earlier) to crosslink HEMA, METC or SPMA monomers. By varying the ratio of monomer to PMeOx crosslinker the elasticity of the beads was varied from *ca.* 2 to 20 kPa to mimic different tissue types. When bone marrow derived human mesenchymal stem cells (hMSCs) were grown on the bead surfaces it was found that without any charged groups (i.e. PMeOx-HEMA) no cell adhesion was observed but with positive (PMETAC) or negative (METAC) charges high numbers of cells attached. Attempts to coat $PMeOx_{30}$-HEMA beads with collagen to improve cell adhesion were unsuccessful due to lack of protein adherence but it did work for the charged systems. To address the issue of toxicity of degradation products (if they were to be transplanted into an animal) the workers used glutathione to cleave the disulfide group of the crosslinker and then studied the molar masses. Structurally, they highlight that the degradation products will contain water soluble PMeOx of low molar mass that should be excreted. SEC measurements confirmed this. Although no animal studies of implanted PAOx materials exist, the *in*

vivo data on soluble materials (Table 1) can still be used to infer if the materials will be well-tolerated in the body.

4.2.2. 3-Dimensional *In Vitro* Models

Three-dimensional (3D) hydrogel *in vitro* tissue models are physiologically more relevant than traditional 2-dimensional flat surface cell culture and can provide pre-animal model data, or eventually replace animal experiments completely. A number of natural and synthetic polymer 3D hydrogels exist each with their own advantages and disadvantages.[98-100] One advantage of using PAOx for *in vitro* models is the possibility of adding biological signalling molecules to the side-groups to promote cell function.[101] One of the first demonstrations that cell adhesion to PAOx hydrogels can be controlled used the ubiquitous RGD integrin binding minimum peptide sequence. The peptide was incorporated into the side-chains by thiol-ene photo-conjugation and photocuring to DecenOx copolymers using cysteine terminated RGD (*viz.* CRGDSG) and dithiothreitol, respectively.[7] In the same study, proof-of-principle experiments showed that it was possible to encapsulate fibroblasts within photocured hydrogels, although multiple time-points were not collected. Similarly, a study examining the MCF-10A epithelial cell line seeded onto PAOx hydrogels with and without RGD peptide showed little cell adhesion without the peptide but good epithelial colony formation on the hydrogels with peptide.[101] An extension of this work with a breast cancer cell line, MDA-MB-231, on non-degradable and MMP-degradable hydrogels showed the spheroid morphology similar to that found in breast cancer could be achieved with the MMP-degradable sequence used to crosslink the hydrogels (Figure 5; unpublished data).

An alternative method for encapsulating cells within PAOx hydrogels was demonstrated by Hickey and co-workers in their work to create artificial female reproductive tract tissue ultimately aimed as a template for studying infection.[70] PMeOx-DecenOx hydrogels were frozen and lyophilized to introduce pores with areas of 200 μm^2 to 3200 μm^2. This porosity range made it possible to seed cells (HFF1 human fibroblast cells and primary fallopian tube stroma cells) on top of the hydrogels and have them infiltrate into the bulk of the hydrogel, hence avoiding any potential DNA dimerization when exposing cells to UV irradiation during photo-encapsulation.

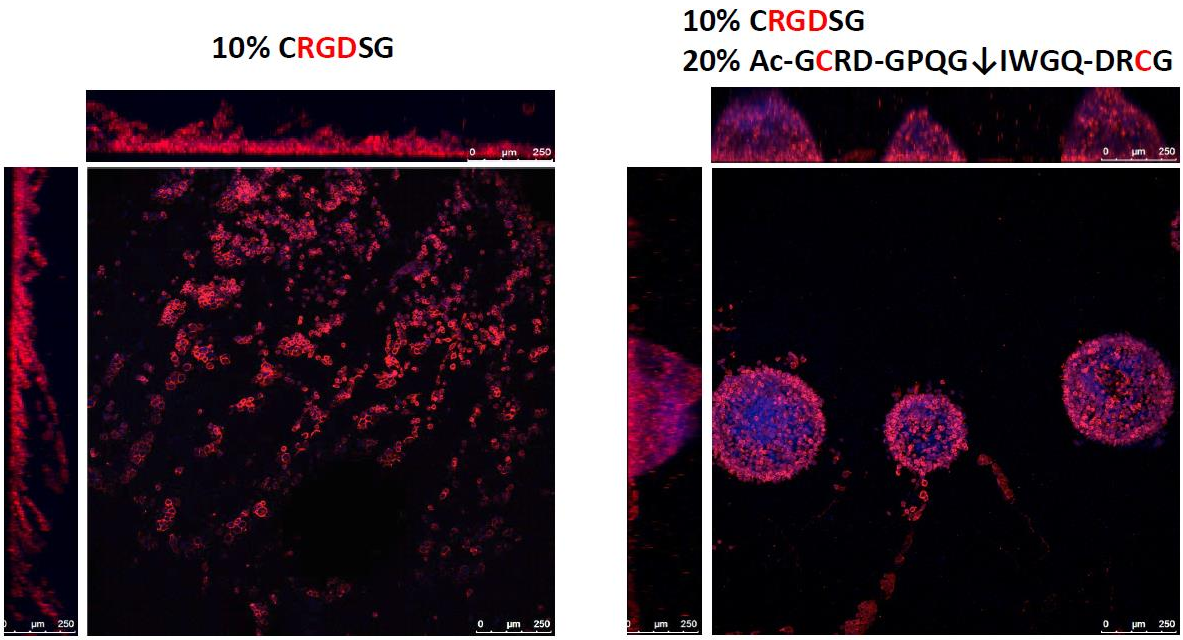


Figure 5. MDA-MB-231 cancer cells grown on top of PMeOx-DecenOx hydrogels without (left) and with (right) an MMP substrate crosslinker. Without the MMP degradable crosslinker the cells spread evenly on the surface but with the MMP degradable crosslinker the cells form spheroids and migrate into the hydrogel as indicated by the z-axis profile on the top and left of each image (unpublished data).

4.2.3 Biofabrication

A new field related to cell therapy and 3D cell culture is biofabrication (also called additive biomanufacturing). Instead of only delivering cells or manually making cell-hydrogel constructs, biofabrication aims to create tissue-like structures aided by a digital input signal. Similar to 'Tissue Engineering' (defined as 'the development of biological substitute to restore, maintain, or improve functions.'[102]), biofabrication involves the generation of biologically functional products using automated bioprinting or bioassembly techniques followed by maturation of the functional product.[103] A technical challenge for biofabrication is the development of 'bioinks' – carrier materials able to be printed in the presence of cells. For this, hydrogels are an obvious choice but the concept of printing hydrogels is not new. For instance, Calvert and Liu reported freeform fabrication of polyacrylamide/acrylic acid/methacrylic acid with silica as a thixotrope 20 years ago.[104] The challenge today is to create printable materials that are also non-toxic, can be loaded with cells, and processed without harming cells (e.g. excess shear stresses).

The group of Luxenhofer have recently reported a 'biocompatible ink' from a triblock copolymer with a poly(2-*n*-propyl-2-oxazoline) middle block and PMeOx outer blocks, described earlier in Section 3.2.[79] This polymer has temperature gelling properties, which was serendipitously discovered when

freezing a sample for lyophilization. Furthermore, the hydrogel has sheer thinning properties making it suitable for extrusion based printing. Small angle neutron scattering (SANS) analysis of the gel suggested an unusual bicontinuous sponge-like structure. What is particularly intriguing about this system is the ability to retrieve the encapsulated cells by lowering the temperature which could be useful if the cells are to be characterization by methods such as flow cytometry. In a proof-of-principle experiment they created a 0°/90° laydown pattern of a single layer of hydrogel/cell composite with good cell distribution and >90% cell viability of NIH-3T3 cells.

Another technique related to biofabrication is melt electrowriting (MEW). Traditionally used with thermoplastic polyesters[105] and polyolefins,[106] MEW has recently been used to make 3D fibrous structures from PEtOx.[107] By heating the polymer to 200-220 °C, well above the T_g, and applying 3-7 kV to charge the polymer, Groll and co-workers were able to achieve sufficient flow of the molten PEtOx to result in electrospun fibres of diameter 8-138 μm. By using a digitally-controlled translating collector they were able to precisely position the fibres to make 3D structures. The study was the first to use MEW with PEtOx and, although not mentioned, the resulting fibres would be presumably dissolve in water given the good water solubility of PEtOx, so some modification would be required to make hydrogels.

One way to make fibrous hydrogels of PAOx was reported by the group of Sanyal.[108] Unlike the MEW work described above, here solution electrospinning was used to create nanofibrous mats of PEtOx-ButenOx mixed with multifunctional thiol crosslinkers and a photoinitiator. Exposure of the electrospun fibers to UV irradiation during spinning resulted in a hydrogel retaining the fibrous features of the electrospun mat when swollen in water. The same approach could conceivably be used with the MEW process or other fused deposition modelling processes, however, these techniques can be demanding on the amount of material needed during printing optimization or filling of dead volumes in the machines. This bottleneck may clear once commercial quantities of functional PAOx become available.

An alternative to MEW or electrospinning to create structured PAOx hydrogels is the use of sacrificial templating. Our group showed that precisely controlled micro-sized channels can be incorporated into PEtOx-ButenOx hydrogels by using a 3D printed sacrificial poly(*ε*-caprolactone) fibrous structure created using MEW.[77] This approach could be used to make hydrogel-based microfluidic devices or tissue engineered constructs with vascular-like features.

4.3 Coatings and Sensors

Earlier in this article it was described how pioneering studies by the group of Textor revealed that under certain conditions, PMeOx functionalized surfaces can exhibit lower protein adsorption when compared to PEG surfaces prepared in the same way.[41] This work has motivated others to examine PAOx coatings for biomaterial and biosensor applications and although they may not necessarily be

defined as classical hydrogels, many of the coatings have hydrogel-like properties in that they are insoluble but highly swollen and may contain some crosslinking, hence their inclusion in this discussion.

What is most striking about the research into PAOx coatings is the variety in findings when examining cell attachment. Some of this can be attributed to the different cell types being used and cell lines versus primary cells, and some of it to the type of PAOx and the preparation method. For instance Davies and co-workers[109] exploited the living nature of PAOx synthesis to conjugate PMeOx, PEtOx, poly(2-isopropyl-2-oxazoline) (PiPropOx) or poly(2-butyl-2-oxazoline) (PButylOx) to amine-functional glass slides by using amino-functionalized glass slides as the terminating group for the CROP. Regardless of the type of polymer used, both epithelial and fibroblasts cell lines were able to grow to confluency. Similarly, Dworak and co-workers showed confluent dermal fibroblasts on PiPropOx and poly(EtOx-2-nonyl-2-oxazoline) on glass at 37 ºC. Interestingly, they were able to remove the cell sheets without enzymatic or mechanical stimulation by dropping the temperature to 20 °C – below the cloud point temperature of the polymers – leading to hydration and swelling of the coating.[110, 111]

The group of Wang have also studied antifouling with the aim of making coatings more stable than PEG *in vivo*.[112, 113] One of their systems was based on block copolymers of PMeOx-poly(4-vinyl pyridine) adsorbed onto silicon wafers and polymethylmethacrylate sheets that were stabilized by crosslinking via hydrogen-bonding of the poly(4-vinyl pyridine) blocks with polyacrylic acid.[112] The block copolymer was synthesized by taking advantage of the direct incorporation of an alkyne group via the use of propargyl-*p*-toluenesulfonate as the initiator for CROP of MeOx followed by coupling the resulting polymer using Huisgen cycloaddition to azide-terminated poly(4-vinyl pyridine) synthesized by atom transfer radical polymerization (ATRP). In their other system, hydroxyl-terminated PMeOx was reacted with hexamethylene diisocyanate to create PMeOx with a terminal isocyanate group to be subsequently coupled to hyperbranched polyethylene imine (PEI).[113] The resulting PEI-*g*-PMeOx was deposited onto solid substrates by mixing with dopamine under alkaline conditions to create a polydopamine-PEI-*g*-PMeOx coating. In both studies with PMeOx-poly(4-vinyl pyridine) or PEI-*g*-PMeOx, the coated substrates were found to have low bovine serum albumin (BSA), platelet and HUVEC adhesion up to 28 days *in vitro*. Similar results were recently reported by the same group for brush copolymers of PMeOx-poly(4-vinyl pyridine) synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization of a PMeOx-methacrylate macromonomer synthesized by capping the CROP of MeOx with methacrylic acid.[114]

An alternative to coating linear polymers to surfaces is to use cyclic polymers. Cyclic PAOx can be prepared based on linear telechelic PAOx synthesized with propargyl *p*-toluenesulfonate as the initiator and 2-azidoethylamine as the terminator followed by intramolecular Huisgen cycloaddition in

dilute solution.[115, 116] When immobilized onto surfaces, the cyclic brushes produced surfaces with higher polymer density and superior protein resistance compared with linear analogues. This example is a clear demonstration of applying elegant polymer synthesis to create intriguing new surfaces.

Partially hydrolyzed PAOx was a theme exploited by Chujo *et al.* in a number of their studies[21, 24, 25, 28, 29] to introduce secondary amines into the PAOx backbone for further modification and is an approach used recently by the group of Konishi to make PAOx gels for sensor applications.[117] They partially hydrolysed a copolymer of PMeOx and PEtOx and subsequently used the resulting ethyleneimine units to conjugate a pyrene derivative and to crosslink using hexamethylene diisocyanate. The resulting gels were amphiphilic and exhibited shifts in fluorescence with changes in swelling, i.e. solvatochromic properties.

The diversity of emerging applications utilizing PAOx highlights the utility and versatile nature of this class of polymers. What is also noteworthy is the number of different groups now working on gels and coatings using PAOx and it will be interesting to see which of these applications will progress to translation. Having considered the work reported to date, in the next section we speculate where the field of PAOx hydrogels will be moving in the coming decade.

5. Future Directions

There has been tremendous interest in using PAOx for self-assembling nanoparticles, drug reservoirs and conjugates in the last decade. Yet, their use in hydrogels is still under-represented. This is despite the advantages of PAOx compared with PEG of: 1) a variety of side chains to modulate hydrophilicity, 2) possibilities to include reactive side-chains, 3) ability to incorporate functional groups to the α and ω termini via judicious choice of initiators and terminating conditions, 4) thermoresponsive properties, and 5) potentially more favourable immuno-response compared with PEG (relevant for *in vivo* applications). Given these attractive features of PAOx for hydrogel synthesis it is worth exploring how the field may evolve in the coming years.

5.1 Polymer Architecture

One area that may see growth is new PAOx hydrogel architectures. Because covalent crosslinking of PAOx through side-chains can result in significant undesired intramolecular reactions it would be of interest to explore other polymer architectures. A recent modelling study of generalized model ‘click’ networks highlighted the interplay between structure and the subsequent ratio of loops to branches after crosslinking.[118] Although block and random copolymers of PAOx are readily achievable, star[119, 120] or hyperbranched systems are less common and almost unused for preparing hydrogels. An exception is a report on crosslinking of star PAOx in 1992 by Chujo *et al.* representing one rare example of using star-shaped PAOx gel precursors.[121] In that work the tri-functional initiators, 1,3,5-tris(iodomethyl)benzene or 1,3,5-tris(*p*-toluenesulfonyloxymethyl)benzene were used in the CROP of

MeOx and terminated with benzylamine. The star polymers were subsequently crosslinked as *ca.* 10% solutions in DMF with hexamethylene diisocyanate and a catalyst. It is important to note that the reactivity of the tri-functional initiators decreased as each electrophile was consumed such that each arm would have been of different length. Furthermore, the terminal benzylamino functionalion degree of each star was poor. This was manifested in the gels which had a maximum yield of 44% and swelling up to nine times in water.

Hyperstars may be an alternative to star polymer gel precursors. Lambermont-Thijs *et al.*[122] capped living chains of PEtOx with amine-functional dendrimers and more recently the group of Perrier[123] reported PAOx hyperstars starting from a tri-allyl core and a thiol-yne monomer. The photo-coupled core was extended with a thiol terminated PEtOx by one pot termination of PEtOx polymerisation with potassium ethyl xanthate followed by aminolysis with dimethylamine to form a thiourea and the mono-thiol-functionalized PEtOx. Their application was drug delivery vectors but it is conceivable that the hyperstars, with some modifications, may have further use in hydrogel synthesis.

5.2 Crosslinking Chemistry

Other than polymer architecture, the crosslinking chemistry is a source of constant innovation. Recent developments in crosslinking chemistry has been discussed earlier in this article, but there remain opportunities to exploit much of the same chemistry as used in self-assembling nanoparticles, as drug reservoirs and conjugates, which could also be used for creating hydrogels. For example, Groll and co-workers[124] recently reported the modification of PMeOx-Butenox/DecenOx copolymers with mercaptothiazolidine using thiol-ene photo-chemistry followed by deprotection to reveal a cysteine group capable to further modification using native chemical ligation with a thioester-containing pentamer or 20-mer. Clearly the same chemistry could be used to create hydrogels with only slight modifications. Likewise, strain-promoted azide–alkyne cycloaddition (SPAAC) is an example of a conjugation tool that has been adapted to create PEG hydrogels[125] and could equally be applied to PAOx with only minor modifications to the previously reported bicyclo[6.1.0]non-4-yne terminated PEtOx.[63] Similarly, glyco-PAOx,[126] POxylated-proteins[127-129] and PAOx-nucleic acids[130] conjugates that were previously reported have potential to be modified for hydrogel synthesis using physical or covalent crosslinking.

5.3 Mechanical Properties

One seldomly mentioned consideration of PAOx hydrogels are their mechanical properties. Most water-rich hydrogels are highly elastic but structurally weak and from personal experience are often too fragile to test in tensile or tear mode. Yet, the area of tough hydrogels is attracting significant attention. Tough hydrogels have been made with other polymer classes including polyacrylamide using the strategy of very long chain-lengths between crosslinks[131] and co-gelation of synthetic polymers with natural polymers (e.g. alginate) to create interpenetrating networks.[132] PAOx

hydrogels that change from gel to liquid with a change in temperature[79] are an example of using structure to add dynamic material properties and so it may be possible to create other PAOx structures to favourably influence other material/mechanical properties.

5.4 Hydrogel Characterization

Characterization methods other than moduli could also be expanded for PAOx hydrogels to better understand the network structure and the relationship between hydrogel properties and gelation method. One challenge in working with hydrogels is dealing with insoluble materials that are not compatible with the regular spectroscopic and spectrometric characterization tools available to the synthetic polymer chemist. Solid state NMR spectroscopy has proven useful by the group of Schubert[71] for their factor VIII protecting PAOx hydrogels to confirm the crosslinking mechanism. They note, however, that the technique is not sensitive enough to quantify the small amount of non-reacted crosslinker. In the same study they also used an innovative endoscope method in their polymerization mixture and show images before and after polymerization showing significant heterogeneity and a yellow gel. Other characterization methods such as SAXS have already been applied to PAOx hydrogels[79] while other techniques such as XPS and ToF-SIMS currently being used for non-hydrogel networks[133] could be adapted for PAOx hydrogels too.

Another important consideration for PAOx hydrogels when used as biomaterials is their biological characterization. It has become an axiom within the field that PAOx is biocompatible, but the long-term studies (both *in vitro* and *in vivo*) are still lacking and will no doubt be completed once products approach commercialization.

5.5 Expanding the Range of Commercially-Available Materials

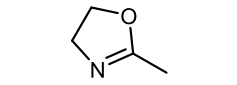
Part of the reason why not more research groups have adopted PAOx is perhaps the lack of commercial monomers and functional polymers. This shortage is gradually being addressed as indicated by Figure 6 and Table 2. The majority of monomers have non-reactive groups in the 2–position, but the range of polarity is quite broad from highly hydrophilic 2-methyl-2-oxazoline to hydrophobic 2-phenyl-oxazoline meaning that a wide range of polymers can be prepared based on (co)polymerization of commercial monomers. There is definitely a shortage of monomers with reactive groups in the 2-position and of the two listed in Figure 6, namely 2-penta-4-ynyl-2-oxazoline and 2-isopropenyl-2-oxazoline, the latter cannot be polymerized to sufficiently high DP due to the resonance stabilization caused by the isopropenyl group[134] although modification prior to polymerization can circumvent this, e.g. radical polymerization of the C=C group followed by CROP of the 2-oxazoline ring[135] or thiol-ene monomer modification followed by CROP.[136]

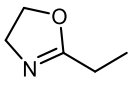
The variety in polarity of the commercially-available polymers (Table 2) is less than the monomers (confined to methyl, ethyl, propyl side-chains) but the reactive end-group functionality is diverse and

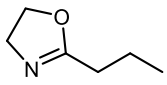
includes hydroxyl, alkyne, azide, amine, thiol and piperazine. Despite the diversity in chemistry, none of these polymers have the degree of functionality required for synthesizing crosslinked networks.

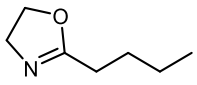
Monomers

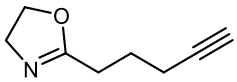
Figure 6. Commercially available monofunctionalized 2-substituted-oxazoline monomers (at the time

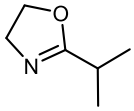

2-Methyl-2-oxazoline

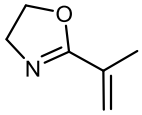

2-Ethyl-2-oxazoline

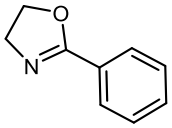

2-*n*-propyl-2-oxazoline


2-*n*-Butyl-2-oxazoline


2-(Penta-4-ynyl)-2-oxazoline

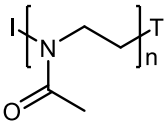
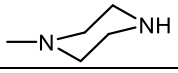
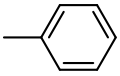
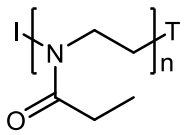

2-Isopropyl-2-oxazoline


2-Isopropenyl-2-oxazoline


2-Phenyl-2-oxazoline

of writing; all from Sigma).

Polymers

Side chain	End-functionality I	End-functionality T	Molar mass (kDa)	PDI	Source
	-CH_3	-OH	5	≤1.3	SA
			10	≤1.2	Ultroxa
	-CH_3	-N_3	10	≤1.2	SA
	-CH_3		10	≤1.2	Ultroxa
		-N_3	2	<1.3	SA
			5	<1.3	
	-CH_3	-	5	3-4	PCI
			15	3-4	SA
			30	3-4	SA
			50	3-4	PCI
			200	3-4	PCI
			500	3-4	PCI
	-CH_3	-OH	5	≤1.2	SA
			10	≤1.3	SA
			10	≤1.2	Ultroxa
			25	≤1.4	SA

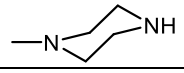
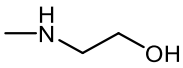
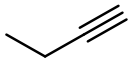
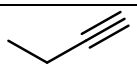
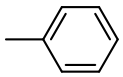
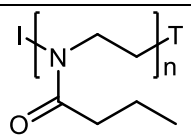
			50	≤1.25	Ultroxa
	$-CH_3$	$-N_3$	10	≤1.2	Ultroxa
	$-CH_3$	$-NH_2$	5	≤1.15	Ultroxa
	$-CH_3$		10	≤1.2	Ultroxa
	$-CH_3$		2	≤1.2	SA
			5	<1.3	SA
			10	<1.3	SA
	-OH		2	≤1.3	SA
			5	≤1.2	SA
		-OH	10	≤1.2	Ultroxa
		-SH	2	≤1.3	SA
			10	≤1.3	SA
	$-CH_3$	-OH	10	≤1.2	Ultroxa
	$-CH_3$	$-N_3$	10	≤1.2	Ultroxa

Table 2. Commercially available PAOx. SA = Sigma Aldrich, Ultroxa = Ultroxa brand distributed through Sigma Aldrich, PCI = Polymer Chemistry Innovations Inc.

6. Conclusions

The synthesis of poly(2-oxazoline)s has flourished in the last couple of decades, but their use in hydrogels is still yet to be fully realized. This is despite the development of 'clickable' PAOx and the possibilities to readily tailor the hydrophilicity/hydrophobicity of the hydrogels simply by changing the monomer selection – a feature not available to many other classes of synthetic polymers. Furthermore, there is an axiom within the field that PAOx are biocompatible based on overwhelmingly favourable *in vivo* data for soluble or nanoparticle forms of the polymer and *in vitro* data on networks and coatings. What is lacking, but probably not far off, is a successfully translated therapy or device based on PAOx that will undoubtedly encourage more research groups to explore this polymer class, including how they can be used to synthesize hydrogels. This, together with the growing literature on synthetic methods for the preparation of functional PAOx and the growing number of commercial monomers and polymers make PAOx hydrogels a fascinating and promising choice of material.

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