Real-time examination of aminoglycoside activity towards bacterial mimetic membranes using Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

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ABSTRACT

The rapid increase in multi-drug resistant bacteria has resulted in previously discontinued treatments being revisited. Aminoglycosides are effective “old” antibacterial agents that fall within this category. Despite extensive usage and understanding of their intracellular targets, there is limited mechanistic knowledge regarding how aminoglycosides penetrate bacterial membranes. Thus, the activity of two well-known aminoglycosides, kanamycin A and neomycin B, towards a bacterial mimetic membrane (DMPC:DMPG (4:1)) was examined using a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). The macroscopic effect of increasing the aminoglycoside concentration showed that kanamycin A exerts a threshold response, switching from binding to the membrane to disruption of the surface. Neomycin B, however, disrupted the membrane at all concentrations examined. At concentrations above the threshold value observed for kanamycin A, both aminoglycosides revealed similar mechanistic details. That is, they both inserted into the bacterial mimetic lipid bilayer, prior to disruption via loss of materials, presumably aminoglycoside-membrane composites. Depth profile analysis of this membrane interaction was achieved using the overtones of the quartz crystal sensor. The measured data is consistent with a two-stage process in which insertion of the aminoglycoside precedes the ‘detergent-like’ removal of membranes from the sensor. The results of this study contribute to the insight required for aminoglycosides to be reconsidered as active antimicrobial agents/co-agents by providing details of activity at the bacterial membrane. Kanamycin and neomycin still offer potential as antimicrobial therapeutics for the future and the QCM-D method illustrates great promise for screening new antibacterial or antiviral drug candidates.

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1. Introduction

The uncontrolled use of antibiotics over almost a century has resulted in bacterial mutations and resistance towards existing treatments. As a consequence, many antibiotics have been rendered ineffective and there is now an urgent need for new classes of antibiotics to combat multi-drug resistant (MDR) organisms [1]. Aminoglycosides were first introduced as antibacterial therapeutics in the 1940’s as they showed activities towards both Gram-negative and Gram-positive bacteria [2]. However, with the emergence of equally potent but less toxic antibiotics, the use of aminoglycosides as antibiotics fell out of favor. But, with the increasing threat of MDR bacteria [3–7] and the decline in new antibiotics approved to help combat this global challenge [8], aminoglycosides are being revisited [9–17]. Because aminoglycosides have been able to largely evade bacterial resistance due to their waned use, it has made them a safer choice [9–17]. In particular, approaches in which aminoglycosides are used in combination therapy with other antibiotics is an emerging strategy [9–18].

Aminoglycosides [19] are a large family of amino-modified sugars, of which streptomycin is the most well-known member [20]. Two related derivatives are kanamycin A and neomycin B. A common structural feature shared by these two aminoglycosides is the presence of the central 2-deoxystreptamine (2-DOs) ring (Fig. 1). In kanamycin, the 2-DOs core is glycosylated with amino sugars at positions 4 and 6, while neomycin features amino sugars at positions 4 and 6, as shown in Fig. 1 [19].

Aminoglycosides are especially active towards Gram-negative bacteria and their primary bactericidal mode of action is through binding to the 16S rRNA component of the 30s subunit of the bacterial ribosome [21–23]. This causes miscoding of genetic material during the translation of mRNA in the 16S rRNA mer, thereby interfering with protein synthesis. Aminoglycosides are also capable of binding to a wide range of other RNA-based structures, including catalytic RNAs.
methyltransferases (conferring class-wide resistance) [12]. Their still side modifying enzymes (mediating selective resistance) and 16S rRNA selves facing an inevitable threat of resistance, mainly from aminoglycoside derivatives in real-time. Aminoglycosides are them -technique enables exploration of the membrane activity of these two with dissipation monitoring (QCM-D) [48,49]. This biophysical branes using a sensitive platform device: a quartz crystal microbalance osides, kanamycin A and neomycin B, towards bacterial mimetic mem-
uptake of aminoglycosides is less well understood. Clearly aminoglycosides to cause liposomal aggregation by binding to anionic lipid head charges among the various aminoglycosides (at physiological pH) indicates that charge may play a role in the interaction of the aminoglycosides with membranes [37–39]. Moreover, the ability of aminoglycosides to cause liposomal aggregation by binding to anionic lipid head groups via electrostatic attraction has also been documented [40–42]. Earlier studies exploring the mode of action of aminoglycosides reported blebs along the cell walls of the Escherichia coli bacteria after aminoglycoside administration [43,44]. More recently, it has been established that aminoglycosides can form channels or fissures in the outer cell membrane [45], allowing their influx into the cytoplasm, followed by targeting of the bacterial ribosomes. Hancock and co-workers have shown that these polycationic aminoglycosides can also be taken up across the E. coli outer membrane by another mechanism termed as self-promoted uptake pathway [38,39,46,47]. Despite significant knowledge of aminoglycoside intracellular activity, the mechanism of uptake of aminoglycosides is less well understood. Clearly aminoglycosides must be able to pass across the biological membrane in order to achieve effective intracellular activity.

In this study, we explore the activity of two well-known aminoglycosides, kanamycin A and neomycin B, towards bacterial mimetic membranes using a sensitive platform device: a quartz crystal microbalance with dissipation monitoring (QCM-D) [48,49]. This biophysical technique enables exploration of the membrane activity of these two aminoglycoside derivatives in real-time. Aminoglycosides are themselves facing an inevitable threat of resistance, mainly from aminoglycoside modifying enzymes (mediating selective resistance) and 16S rRNA methyltransferases (conferring class-wide resistance) [12]. Their still remain outstanding questions on the mode of action of aminoglycosides [9,11–15]. New aminoglycoside derivatives, chemically modified to circumvent resistance, can be foreseen to play an important role as antibiotics in the future [9,11–15]. The data provide additional insights that may assist medicinal chemists with the development of this next generation of aminoglycoside-based antibacterial therapeutics.

2. Experimental section

2.1. Chemicals

Neomycin B trisulfate, kanamycin A sulfate, sodium chloride, potassium phosphate monobasic and potassium phosphate dibasic, cholesterol, chloroform (≥99.8%) and methanol (≥99.9%) were all purchased from Sigma-Aldrich (St. Louis, USA) and used without purification. The synthetic phospholipid derivatives, 1,2-dimyristoyl-snglycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycerol-3-phospho-rac-(1-glycerol) (sodium salt) (DMPG), were purchased from Avanti Polar Lipids (Alabaster, USA) and 3-mercaptopropion acid (MPA) from Fluka. Ultrapure water with an initial resistivity of 18.2 MΩ cm was used for all experiments. Phosphate buffered saline solutions (20 mM KH2PO4 and K2HPO4, pH 6.9 ± 0.1) containing either 100 mM (high-salt PBS) or 30 mM (low-salt PBS) sodium chloride were prepared in water.

2.2. Instrumentation and methods

A lipid composition of DMPC/DMPG (4:1 v/v) was used for liposome preparation (see Supporting Information for details), following our previously published procedure [50,51]. The Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D) measurements were performed using E4 system with flow cells (Q-Sense, Västra Frölunda, Sweden). The polished, gold-coated, AT-cut quartz chips with a fundamental oscillating frequency of ca. 5 MHz, were used as sensor crystals. The sensors were treated with mercaptopropion acid to create a self-assembled carboxylate monolayer prior to the introduction of the liposomes as described in the Supporting Information. All QCM-D experiments were conducted at 19.10 ± 0.05 °C [52], in triplicate, using established experimental protocols also used for peptides–membrane interactions (see Supporting Information for details) [51, 53–55]. Concentration studies were carried out using 1, 10 and 15 μM solutions of kanamycin A and neomycin B in high-salt PBS. For a typical QCM-D experiments, the relative changes in resonance frequency (Δf)
and energy dissipation (ΔD) of the sensor were simultaneously recorded at the 1st, 3rd, 5th, 7th and 9th harmonics. The primary Δf − t and ΔD − t QCM-D data were analyzed using OriginPro 8 (OriginLab, Northampton, USA) and Δf vs ΔD graphs were used for further analysis. Data for the 1st harmonic (i.e., fundamental frequency of the crystal) were not included in the analysis as it is generally unreliable, being influenced by the flow of the solution through the QCM chamber [55]. For discussion purposes, only the 7th harmonic data plots have been presented, unless otherwise stated. A typical experiment is illustrated in Fig S1.

3. Results and discussion

The interaction of kanamycin A and neomycin B with phospholipid bilayers consisting of DMPC/DMPG (4:1) was analyzed using the QCM-D. The anionic DMPC/DMPG (4:1) lipid combination is recognized as being a good biomimetic for the prokaryotic cell membrane [56,57], particularly the Gram-positive bacterial membrane [58,59]. The frequency change (Δf) from the QCM-D is related to the change in mass [60], while the dissipation output (ΔD) can be used for the qualitative profiling of structural changes in the bilayer. A decrease in Δf value implies the addition of mass onto the sensor, whereas an increase in ΔD indicates that the lipid bilayer is becoming less rigid, loosening or thickening [60–63].

The effect of the addition of kanamycin A on the lipid membrane was followed by measuring the Δf vs. t data, which showed the concentration dependence depicted in Fig. 2. The data was normalized prior to the addition of the aminoglycoside. When introduced into the chamber at 1 μM concentration, kanamycin A was found to slowly add onto the phospholipid membrane. As can be gauged from the respective Δf − t traces (Fig. 2), the oscillation frequency of the sensor decreased with time upon addition of kanamycin A until all the solution was added (~17 min). After this time, the flow was ceased and the samples were incubated for 30 min. At higher concentrations (10 and 15 μM), however, rapid removal of material from the membrane-coated sensor occurred after the initial mass addition to ~5 Hz. At these higher concentrations the threshold for the initial binding of kanamycin A shows a decrease in mass (~4–5 Hz) was similar, however a second ‘disruption’ phase corresponding to a greater mass removal from the lipid membrane was observed at 15 μM, suggesting a concentration dependence.

A similar biphasic trend of rapid initial mass uptake and material removal thereafter was observed for neomycin B, at all the concentrations studied (Fig. 3). In contrast to kanamycin, however, there was no concentration threshold observed, although the process was characterized by insertion to 4–5 Hz, followed by disruption. Interestingly, as observed for kanamycin A, membrane disruption was greatest at the highest concentration studied (15 μM), as shown in Fig. 3.

QCM-D experiments are also able to provide depth profiling via analysis of frequency changes at different harmonics of the QCM-D sensor [51,53–55]. Each harmonic probes a defined distance away from the surface of the sensor. This distance is inversely proportional to the frequency of the harmonic [64]. For example, the higher harmonics (7th and 9th) probe close to the sensor surface, whereas the lower harmonics (3rd and 5th) probe further away. Thus, probing the harmonics in a QCM-D experiment can provide a method to assess the nature of the interaction of the aminoglycoside with the membrane layer, i.e., surface vs. trans-membrane binding. The spread of the harmonics (from the 3rd to the 9th) in Δf − t and ΔD − t plots for neomycin B (1–15 μM) and kanamycin A (10 and 15 μM) was similar, as illustrated in Fig. 4 where the 3rd, 5th, 7th and 9th harmonics are shown. This initial overview illustrates the similarities between kanamycin and neomycin in terms of their interaction with the membrane layer. The Δf − t data for kanamycin (Fig. 4a) and neomycin (Fig. 4b) both show a decrease in frequency to ~5 Hz, with overlap of the harmonics. Thus, the initial phase of the aminoglycoside–membrane interaction is independent of the distance from the sensor, consistent with mass addition equally across the membrane, i.e., in a trans-membrane fashion. However, in the second phase, beginning at ~5 min, there is the loss of mass (Δf increases) from the sensor, which is characterized by a differential response depending on the harmonic used to sense the frequency. Thus, the 3rd harmonic, which probes the furthest distance, shows the largest increase in frequency, whereas the 9th harmonic plots displayed the lowest increments (Fig. 4). In the second phase, more mass is being removed from the surface of the membrane.

Similar trends were observed for the ΔD − t plots shown in Fig. 4(c) and (d). In these cases, the largest decrease in dissipation was observed for the 3rd harmonic, with progressively smaller decreases in dissipation energy found for higher harmonics. These decreases in ΔD are consistent with a loss of membrane (or aminoglycoside–membrane materials), since the sensor would be expected to become less viscoelastic in response to membrane loss. The data provided by the different harmonics indicate more material being removed from the membrane surface with time, resulting in a decrease in membrane thickness.

Returning to the kanamycin data obtained at 1 μM concentration, only a small displacement of the 3rd harmonic is observed, with the other harmonics almost overlapping (Fig. 5). As discussed above for the initial process, the overlapping harmonics suggest that the added aminoglycoside is inserting in a trans-membrane manner, however the displacement of the 3rd harmonic suggests that the surface of the membrane is affected slightly differently. There is always the possibility...

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**Fig. 2.** Δf−t traces from QCM-D monitoring of the interaction of kanamycin A with a DMPC/DMPG (4:1) membrane showing the effect of concentration on membrane uptake.

**Fig. 3.** Δf−t traces from the QCM-D monitoring of the interaction of neomycin B with a DMPC/DMPG (4:1) membrane showing the effect of concentration on membrane uptake.
that the membrane surface is somewhat irregular. For example, embedded liposomes could be present that did not burst during the membrane deposition. However, the small but clear harmonic effect observed in the $\Delta D - t$ values suggests that a two-phase process similar to that observed with the higher aminoglycoside concentrations is occurring. Thus, the responses of the harmonics shown in Fig. 5b (maxima followed by decreases in $\Delta D - t$ values) are consistent with insertion of kanamycin into the membrane, albeit with slightly more binding occurring at the surface of the membrane, followed by a small loss of material from the surface and a loosening of the membrane layer. It appears that the data in Fig. 5 were captured at just the right concentration for the concentration threshold interaction to be observed.

The temporal frequency and dissipation changes can be combined as $\Delta f - \Delta D$ traces to provide greater insight into changes in the structural integrity of the lipid bilayer occurring during mass addition/removal processes. We have previously shown that these traces provide a unique “fingerprint” characteristic of the mode of interaction of a small molecule or biomolecule with the supported phospholipid bilayer-modified sensor [51,53,54,65]. The $\Delta f - \Delta D$ fingerprints for both kanamycin A and neomycin B are shown in Fig. 6 (7th harmonic data shown only).

In these plots, the origin represents the starting point for both aminoglycosides. Initially, the binding of each compound is indicated by the observed frequency decrease, i.e., the trace moves from the origin to a maximum value, or ‘turning point’, that appears characteristic of

![Fig. 4. QCM-D monitoring of kanamycin A (10 μM); (a) $\Delta f$-t; (c) $\Delta D$-t plots, and neomycin B (10 μM); (b) $\Delta f$-t; (d) $\Delta D$-t traces, showing the uptake on a DMPC/DMPG (4:1) bacterial mimetic membranes. The response of the 3rd, 5th, 7th and 9th harmonics of the QCM-D sensors are each shown in these panels.](image)

![Fig. 5. QCM-D monitoring of kanamycin A (1 μM) uptake on a DMPC/DMPG (4:1) membrane with (a) $\Delta f$-t and (b) $\Delta D$-t plots showing the effect on the 3rd, 5th, 7th and 9th harmonics.](image)
each aminoglycoside (its concentration threshold). These traces then
almost reverse in direction and continue towards the lower quadrant
(positive frequency and negative dissipation region), indicating a loss
of materials from the sensor. In summary, the interaction between
these aminoglycosides and the model phospholipid membrane is
biphasic in nature. The initial decrease in Δf with minimal changes in
ΔD values, followed by a large decrease in ΔD along with an increase
in Δf values is characteristic of trans-membrane insertion followed by
membrane disruption.

Qualitative evaluation of the rates of association/dissociation of
kanamycin A and neomycin B was also carried out via time dependent
analysis of the first-order derivative of the Δf- t curves (∂Δf/∂t vs. t).
These kinetic profiles are shown in Fig. 7 for two concentrations
(1 and 10 μM) of each of the aminoglycosides and over the period of
time in which material addition and removal processes occurred. These
derivative traces reveal an extremely slow mass uptake (<1 Hz/min)
of kanamycin A at 1 μM concentration, with the rate of mass addition
increasing in the order kanamycin A (1 μM) ≪ neomycin B (1 μM) < kanamycin A (10 μM) ≈ neomycin B (10 μM). At the higher
concentrations of kanamycin A and neomycin B, removal of the material
from membrane was ca. 3-fold faster for neomycin B compared to
kanamycin A. Interestingly, at 10 μM concentration, the initial insertion
step occurred at a similar rate for both aminoglycosides (-7 Hz/min),
whereas the disruption that followed was faster for neomycin B
(-20 Hz/min) compared to kanamycin A (-7 Hz/min). The data for
neomycin B at 1 μM also showed a similar trend, with the rate of
disruption approximately two times greater than the rate of insertion
into the membrane.

Both kanamycin A (+4) and neomycin B (+6) are positively
charged at pH = 6.90. Thus, the initial rapid association and insertion
into the lipid membrane could be due to strong electrostatic attraction
with the anionic membrane surface (DMPC/DMPG (4:1)). In addition,
the DMPG component contains a glycerol group that could assist in
hydrogen-bonding interactions with the cationic aminoglycosides,
although we have no direct evidence of these interactions. The associa-
tive binding of the aminoglycosides is not a favorable interaction in the
membrane, and we believe that local disorder in the lipid bilayer results,
followed by removal of material. Thus, disruption of bacterial mimetic
DMPC/DMPG (4:1) membrane by kanamycin A and neomycin B is a
biphasic process, as illustrated in Fig. 8 [53–55,66]. The individual
polycationic aminoglycoside molecules insert into the membrane
surface in a trans-membrane orientation, until a threshold concentra-
tion is reached, whereupon the combined interactions between the
molecules and the lipid membrane (electrostatics, hydrogen-bonding)
result in destabilization of the membrane and accretion of material.

4. Conclusion

QCM-D analysis indicates that the two well-known aminoglyco-
sides, kanamycin A and neomycin B, interact with a bacterial mimetic
membrane (DMPC/DMPG (4:1)) via a two-stage mechanism. Initial
aminoglycoside insertion into the membrane is rapid and occurs in a
trans-membrane fashion. This is followed by an equally facile mem-
brane disruption stage once a critical concentration threshold is
reached. Loss of membrane material occurs more from the surface of
the membrane than the interior. Kanamycin A and neomycin B both
show similar rates of insertion into the anionic membrane surface,
however they differ slightly in their rates of membrane disruption.
Neomycin B causes a greater degree of membrane disruption, which is
argued to be due to its greater cationic charge. It is hypothesized that
electrostatic interactions of the polycationic aminoglycoside with the
anionic membrane surface and intermolecular hydrogen-bonding inter-
actions between the lipid membrane and the charged aminoglycoside
molecules contribute to local disorder and stress within the lipid bilayer,
leading to the membrane damage and removal of material. If this is
indeed the case, incorporation of functional groups to enhance these
interactions could produce compounds with enhanced potency, which
might serve as the basis for new antimicrobial therapeutic treatments
urgently needed to combat the rapid growing population of drug-
resistant viruses and bacteria. With this in mind, we are currently

![Fig. 6. Energy dissipation (ΔD) vs. frequency (Δf) dependence of the interaction of (a) kanamycin A and (b) neomycin B with a DMPC/DMPG (4:1) membrane. The x- and y-axis represent Δf and ΔD (10^-6) values, respectively.](image)

![Fig. 7. First-order derivative of 7th harmonic Δf-t traces vs. time for the interaction of 10 μM kanamycin A and neomycin B with a DMPC/DMPG (4:1) membrane, as plotted against time. Data for time ≥ 20 min has been omitted for clarity.](image)
conducting in-depth QCM-D investigations of the interactions of several such modified aminoglycoside derivatives with bacterial mimetic membranes.

Abbreviations

**DMPC** 1,2-dimyristoyl-sn-glycero-3-phosphocholine

**DMPG** 1,2-dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol)

**DOS** 2-deoxystreptamine

**MDR** Multi-drug resistance

**MPA** 3-mercaptopropionic acid

**QCM-D** Quartz Crystal Microbalance with Dissipation monitoring

**RNA** Ribonucleic acid

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.10.019.

References


Highly potent antimicrobial polyionenes with rapid killing kinetics, skin biocompatibility and in vivo bactericidal activity

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Abstract

Effective antimicrobial agents are important arsenals in our perennial fight against communicable diseases, hospital-acquired and surgical site multidrug-resistant infections. In this study, we devise a strategy for the development of highly efficacious and skin compatible yet inexpensive water-soluble macromolecular antimicrobial polyionenes by employing a catalyst-free, polyaddition polymerization using commercially available monomers. A series of antimicrobial polyionenes are prepared through a simple polyaddition reaction with both polymer-forming reaction and charge installation occurring simultaneously. The compositions and structures of polymers are modulated to study their effects on antimicrobial activity against a broad spectrum of pathogenic microbes. Polymers with optimized compositions have potent antimicrobial activity with low minimum inhibitory concentrations of 1.95–7.8 mg/mL and high selectivity over mammalian cells. In particular, a killing efficiency of more than 99.9% within 2 min is obtained. Moreover, the polymers demonstrate high antimicrobial efficacy against various clinically-isolated multidrug-resistant microbes, yet exhibit vastly superior skin biocompatibility in mice as compared to other clinically used surgical scrubs (chlorhexidine and betadine). Microbicidal activity of the polymer is mediated via membrane lysis as demonstrated by confocal microscopy. Unlike small molecular antibiotics, repeated use of the polymer does not induce drug resistance. More importantly, the polymer shows excellent bactericidal activity in a P. aeruginosa-contaminated mouse skin model. Given their rapid and efficacious microbicidal activity and skin compatibility, these polymers have tremendous potential to be developed as surgical scrubs/hand sanitizers to prevent multidrug-resistant infections.

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1. Introduction

Since penicillin was discovered, many antibiotics have been identified that exert antibacterial effects by inhibiting various targets within microbial biosynthetic pathways [1–3]. An unexpected drawback of the high therapeutic specificity of these antibiotics is the subsequent development of drug resistance mediated through sequential mutations [2,4,5]. Antimicrobial compounds are extensively used in personal care products to prevent infections and prolong product shelf-life [6–8]. Most antimicrobial agents found in personal care goods have molecular weights below 600 Da and include triclosan, chlorhexidine and benzalkonium chloride. Triclosan is amongst the most extensively used compounds and is present in many personal care products such as cosmetics,

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deodorant and soap [9,10]. Though efficacious against Gram-positive bacteria, its effectiveness against Gram-negative bacteria and mould has been found wanting [9,11,12]. For the antimicrobial agents as aforementioned, there has been documented resistance against various strains of bacteria. More importantly, there is a major concern with the development of cross- and co-resistance with clinically-used antibiotics, further complicating the resistance dilemma.

Therefore, there exists an urgent need to discover more effective antimicrobial agents against multidrug-resistant (MDR) pathogens, which would mitigate drug resistance development. In spite of the pervasive exploitation of cationic antimicrobial peptides (AMPs) in our innate immune system, these AMPs have still been largely protected from the development of bacterial resistance [13]. This is due in large part to the fact that these AMPs utilise less selective cationic amino acids which interact with bacterial membranes electrostatically. Secondary conformational changes, which occur after electrostatic membrane association, allow the hydrophobic regions within these AMPs to integrate with the bacterial cell membranes [14,15]. These cell membranes are destabilised once these hydrophobic regions are inserted into the lipid bilayers, eventually resulting in cell lysis and death. As opposed to antibiotics that inhibit specific intracellular microbicide processes, these AMPs exert their antimicrobial activity via non-specific electrostatic and hydrophobic interactions. This unique mode of action largely shields AMPs from the development of the antimicrobial resistance.

A number of synthetic cationic polymers that mimic the amphiphilic structure and antimicrobial functionalities of AMPs have been explored as highly tunable and broad spectrum antimicrobials [16–18]. For examples, polycrlylates [19–23], poly-norbornene systems [23], polyethyleneimines [24,25], polyarylamides [27], metallopolymers [26], poly-β-lactams [28,29], and polycarbonates [30,31] have shown minimum inhibitory concentration (MIC) values of 4 to >1000 μg/mL. In general, the overall hydrophobic/hydrophilic balance of the polymers affects their antimicrobial activity and bacteria selectivity [32,33]. An alternative class of polymer electrolytes is the polionenes, wherein the quaternized nitrogens are located in the polymer backbone in contrast to the above examples where the distal charges are pendent to the backbone [34,35]. Charge density and hydrophobicity of polionenes can be readily tuned as compared to polycarbonates and polycrlylates by choosing appropriate commercially available monomers [36,37]. Polionenes have been studied as antimicrobials. For example, Agarwal and coworkers investigated alklyoxyethylammonium ionenes with varying pendant groups and alkyl spacers in the main chain as biocides [38]. By employing polyaddition reaction of N,N,N',N' tetramethyldiamines and α,ω-dibromoalkanes, Tiller's group also found that such polionenes had excellent antimicrobial activity, and were not hemolytic towards red blood cells [39]. In general, the antimicrobial efficacy of polionenes has been shown to be heavily dependent upon chain rigidity and alkyl groups of varying length, which were used to modulate hydrophobicity [38,39]. Our group has recently designed a class of antimicrobial polycarbonates and found that use of long alkyl chains led to high toxicity towards mammalian cells, while use of aryl group or cyclic group mitigated toxicity [33,40]. Moreover, there are limited studies reported on activity of polionenes against clinically-isolated MDR microbes, skin biocompatibility and in vivo bacterial activity. Therefore, in this manuscript, aryl groups were used in the synthesis of polionenes to optimize hydrophobicity for high potency and in vivo skin biocompatibility.

Specifically, a new series of antimicrobial polionenes has been synthesized through a simple polyaddition reaction, whereby both the polymer-forming reaction and the installation of the charge occur simultaneously (see Scheme 1, polymers 1a–1c, and polymers 4a–4c). Moreover, the use of commercially available, low-cost starting materials, as well as simplified polymerization and isolation conditions are anticipated to lower the cost of final products. The objective of this study is to focus on efficacy and killing kinetics. The compositions and structures of the polymers were modulated to study their effects on antimicrobial activity against pathogenic microbes, including S. aureus, E. coli, P. aeruginosa and C. albicans, and their selectivity towards mammalian cells (e.g. rat red blood cells). The killing kinetics of the polymers were investigated, and the antimicrobial mechanisms were explored by visualizing the changes in the membrane integrity of microbes after polymer treatment. Possibility of drug resistance development in bacteria was evaluated by monitoring MIC changes after repeated use of polymers at sub-lethal doses. In addition, the antimicrobial activity of the polymer with the optimized composition was examined against clinically isolated multidrug-resistant bacterial strains including multidrug-resistant S. aureus, MRSA, E. coli, A. baumannii and K. pneumoniae, as well as fungi C. albicans and C. neoformans. Finally, the in vivo bacterial activity and dermal toxicity of the polymer were evaluated using a mouse model.

2. Materials and methods

2.1. Materials

All chemical reagents were supplied by Sigma-Aldrich, U.S.A. and used as received unless specified otherwise. Micorbial broth samples were prepared using Muller Hinton Broth (MHB) powder (BD Diagnostics). S. aureus (ATCC No. 6538), E. coli (ATCC No. 25922), P. aeruginosa (ATCC No. 9027) and C. albicans (ATCC No 10231) were obtained from ATCC, U.S.A. Clinically isolated multidrug-resistant Gram-positive bacteria S. aureus and MRSA, Gram-negative bacteria E. coli, A. baumannii and K. pneumoniae, and fungi C. albicans and C. neoformans, were extracted from patients’ phlegm or cerebrospinal fluid (multidrug-resistant C. neoformans) and kindly provided by Dr. JiJang Sheng, Department of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, P. R. China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was prepared in phosphate-buffered saline (PBS, pH 7.4) at 5 mg/mL. This solution was subsequently subjected to filtration using a filter (0.22 μm) so as to remove blue formazan crystals before usage. Fetal bovine serum was procured from Invitrogen Corporation. Rat red blood cells (RBCs) from Vistar rats (Invisos, Singapore) were employed for hemolysis study. Primary human dermal fibroblasts (HDFs) were obtained from ATCC (PCS-201-012).

2.2. Synthesis of polymers

The synthesis of polymer 4 is given as an example. To a round-bottom flask equipped with a nitrogen inlet was added 1,4-bis(chloromethyl)benzene (1.65 g, 9.43 mmol) and DMF (20 mL). A solution of bis[2-[(N,N-dimethylamino)ethyl] ether (1.51 g, 9.43 mmol) in DMF (20 mL) was then added dropwise over 30 min at room temperature. The reaction is exothermic. After the addition was complete, the reaction mixture was heated to 85 °C and stirred for 18 h, at which point a precipitate was observed. The polymer was isolated by filtration and washed with ethyl ether (3 × 50 mL) to afford an off-white powder in near quantitative yield.

1H NMR (400 MHz, D2O): δ 7.58 (s, 4 H, PhH), 4.52 (s, 4 H, PhCH2), 3.98 (s, 4 H, CH2CH2N+(CH3)2), 3.59 (s, 12 H, -N+(CH3)2), 3.01 (s, 12 H, -N+(CH3)2), M ~ GPC = 7930 Da, D = 2.6.
Scheme 1. Direct polyaddition and concurrent introduction of charge in a single step. Counteranions are omitted for clarity.
2.3. Molecular weight determination

A Waters 2695 gel permeation chromatography (GPC) equipped with two ultrahydrogel columns: 300 mm × 7.8 mm in series and a Waters 2414 differential refractometer detector (MA, U.S.A.) were used to determine the molecular weights of the polymers synthesized. Details of the mobile phase used are as follows: 54:23:23 (v/v/v) water/methanol/acetic acid, 0.5 M sodium acetate at a flow rate of 1.0 mL/min. Calibration was performed using a series of poly(ethylene glycol) standards of varying molecular weights (633–20,600) (Polymer Standard Service Inc., RI, U.S.A.). $M_w$ and $D$ was subsequently calculated from the calibration curve.

2.4. Antimicrobial activity

2.4.1. MIC measurement

Bacterial and fungal samples were grown in MHB at 37 °C and room temperature, respectively, under shaking (100 rpm). They were subsequently incubated overnight so as to enter the log growth phase. The respective MIC of each polymer was determined using a broth microdilution method. Each microbial suspension (100 μL) was seeded into each well of a 96-well plate ($3 \times 10^5$ colony forming units (CFU) mL$^{-1}$), to which 100 μL of broth containing a polymer at different concentrations was added. The plate was then subjected to incubation under shaking (100 rpm) for 18 h at 37 °C. MIC was taken to be the lowest polymer concentration at which microbial growth was completely inhibited by observation with a microplate reader (TECAN, Switzerland). Negative controls (broth containing only microbes without polymer treatment) were used. Six replicates were repeated for each experiment. The experiment was independently repeated at least three times.

2.4.2. Drug resistance study

E. coli were used as a model microbe for the drug resistance study. Drug resistance was induced by repeatedly treating E. coli (ATCC No 25922) with antimicrobial agents at sub-lethal doses [41]. The MIC of polymer 1 against E. coli was determined through 10 passages of growth. MIC was determined using the broth microdilution method described above. E. coli exposed to sub-MIC concentrations (1/8 of MIC at that particular passage) were allowed to re-grow and reach a logarithmic growth phase before being used for MIC measurement of the subsequent passage. Development of drug resistance in E. coli was evaluated by recording changes in the MIC normalized to that of the first cell passage.

2.5. Killing kinetics test

The samples were then subjected to centrifugation (10000 g, 5 min at 4 °C). The supernatant (100 μL) was then pipetted into each well (96-well culture plate), and 4 replicates were repeated for each polymer concentration. The optical density of each well was then recorded at 576 nm using the microplate reader. This approximates to the amount of hemoglobin released and the degree of hemolysis of each polymer. Negative (rRBC suspension without polymer treatment) and positive (rRBC suspension with addition of 0.1% Triton-X) controls were used. The formula below was used to calculate the degree of hemolysis:

\[
\text{Hemolysis} = \frac{\text{O.D.576 nm of the treated sample} - \text{O.D.576 nm of negative control}}{\text{O.D.576 nm of positive control} - \text{O.D.576 nm of negative control}} \times 100%
\]

Hemolysis was taken as polymer concentration at which the polymer causes 50% hemolysis. The experiment was independently repeated three times.

2.8. Water-octanol partition coefficient (log P)

Polymer solutions (500 μL, 125 μg/mL) were prepared in aqueous solution and an equal volume of octanol was then added. The samples were vortexed for 10 min and then allowed to equilibrate overnight in the dark. The samples were then centrifuged at 4000 rpm for 5 min. 100 μL from each phase was transferred to a new tube and diluted 10-fold in methanol. Their corresponding UV–Vis spectra were recorded. The polymer concentration in each phase was determined with reference to calibration curves obtained by measuring the UV–Vis spectra of various concentrations of each polymer in methanol. The partition coefficient was defined as log $P = \log([C]_{\text{oct}}/[C]_{\text{aq}})$, where $[C]_{\text{oct}}$ and $[C]_{\text{aq}}$ are the polymer concentrations in octanol and aqueous phases, respectively. When log $P$ is lower than 0, a polymer is considered to be relatively hydrophilic. Measurements were conducted in triplicates.

2.9. In vitro cytotoxicity study

2.9.1. MTT assay

The MTT assay was performed to evaluate the cytotoxicity of the polymers. HDF cells (10$^4$, 100 μL) were seeded into each well in 96-well plates. They were then cultured in the standard medium (DMEM supplemented with 10% FBS, 5% penicillin-streptomycin, 2 mM L-glutamine, 4.5 g/L α-glucose and 110 mg/L sodium pyruvate), and incubated at 37 °C, 5% CO$_2$ overnight. The samples were then subjected to incubation at 37 °C in 5% CO$_2$ for 2 min. After treatment, the solutions within each well was removed, and subsequently replaced by 100 μL of fresh medium and 20 μL of MTT solution (5 mg/mL). After incubating for 4 h at treatment. This was followed by re-suspension of the pellet in PBS and staining with PI (30 μM) or FITC-dextran (250 μg/mL, 100 kDa) at room temperature for 15 min. After being washed twice with PBS, the cells were observed under CLSM (Carl Zeiss LSM 510 META inverted confocal microscope, Germany). Similar conditions were maintained for all experiments.
37 °C in 5% CO₂, the medium within each well was then replaced with dimethyl sulfoxide (150 μL). Using a microplate reader, absorbance was then measured at 550 nm, with the absorbance at 690 nm taken as a reference. Cell viability was expressed as a percentage of absorbance of the control cells without any treatment. The experiment was independently repeated three times.

2.9.2. LDH (lactate dehydrogenase) assay

The LDH assay (Promega, U.S.A.) was performed to quantitatively measure the amount of LDH enzyme released by cells with damaged or lysed membrane as an indicator of cytotoxicity. HDF cells (10⁴ cells per well in 96-well culture plates) were treated with polymer 1 or chlorhexidine digluconate at various concentrations for 2 min or 1 h (8 replicates for each concentration), and the supernatant (50 μL) was then transferred into the wells of a new plate. Freshly prepared LDH assay solution (50 μL) was added, and the plate was incubated for 30 min at room temperature according to the manufacturer’s protocol. This was followed by the addition of 50 μL of Stop solution. The optical density at 490 nm of each well was then recorded using the microplate reader. This approximately corresponds to the amount of LDH released and the cytotoxicity of each polymer concentration. Negative (cells without polymer treatment) and positive (cells completely lysed in 1 × lysis solution) controls were used. The formula below was used to calculate the cytotoxicity of the polymer and chlorhexidine,

\[
\text{Cytotoxicity (\%) = } \frac{[(\text{O.D. } 490 \text{ nm of the treated sample } - \text{O.D. } 490 \text{ nm of negative control})]}{[(\text{O.D. } 490 \text{ nm of positive control } - \text{O.D. } 490 \text{ nm of negative control})]} \times 100\%
\]

2.10. Animal studies

The animal study protocols were approved by the Institutional Animal Care and Use Committee of Biological Resource Centre, Agency for Science, Technology and Research (A*STAR), Singapore.

2.10.1. Animal skin toxicity evaluation

In vivo dermal compatibility studies were performed with C57BL/6 mice (8 weeks old, 18–22 g). The mice were randomly grouped (5 in each group). Before the test, fur in the dorsal area (about 1.5 cm × 1.5 cm) of each mouse was removed. The mice were then treated with 6 topical solutions: water solution (control), chlorhexidine (cotton, control, 0.198% w/v), chlorhexidine (neutral control, 4% w/v in Bactishield handwash), betadine (control) and polymer 1 (200 and 500 μg/mL). 0.198% w/v was used for benzalkonium chloride as it is the effective concentration of benzalkonium chloride in Dettol handwash. The samples (200 μL) were administered topically and uniformly on the shaved area of each mouse. After exposure for 2 min, the treated skin area was rinsed with water. The mice were treated twice daily for 5 days total. The mice were sacrificed and the treated skin tissues were collected for histological examination. This involved the fixation of samples in 4% neutral buffered formalin followed by paraffin, and subsequent sectioning and staining with hematoxylin (nucleus, blue) and eosin (cytoplasm, purple) under standard protocols.

2.10.2. In vivo toxicity studies

LD₅₀ was determined using female Balb/c mice (8 weeks old, 18–22 g). The Up-And-Down-Procedure described in OECD Guidelines for the Testing of Chemicals (OECD 425) was used to determine LD₅₀. Polymer 1 was dissolved in water and administered to mice orally at various doses (i.e., 1000, 1250, 1500, 1750 and 2000 mg/kg, 0.2 mL). Mortality was monitored for 14 days post treatment, and the maximum likelihood method was used to estimate LD₅₀.

2.10.3. In vivo bactericidal activity

A mouse model was established using a previously reported method [42] to study the bactericidal activity of polymer 1. C57BL/6 mice (8 weeks old, 18–22 g) were employed for this study. The mice were randomly grouped (5 in each group). Mice were anesthetized using an i.p. injection with pentobarbital (50 mg/kg). Once the mice were fully anesthetized, the hair of the dorsal area was clipped and the depilatory cream was introduced to completely shave off the dorsal area. The test area (2 cm²) on the back of each mouse was disinfected with cotton soaked in ethanol, and allowed to dry for 30 min. Suspension of clinically isolated P. aeruginosa 25844 (10 μL, 5 × 10⁶ CFU/mL) was applied to the dorsal area, and then dried for 30 min. Polymer 1 (500 μL, 500 μg/mL) and chlorhexidine (500 μL, 4% w/v in Bactishield handwash) were administered topically on the bacteria-contaminated sites. After 2 min of treatment, the full-thickness skin of the application site was removed under anaesthesia using sterile forceps and scissors, and placed in 5 mL of MHB in order to neutralize the bactericidal activity of the test compounds. The skin of untreated mice in the control group was recovered using the same protocol. The MHB was plated on agar plates. CFUs were subsequently counted after 24 h of incubation.

2.11. Statistics

The results are expressed as mean ± standard deviation. Standard deviation is indicated by the error bars. Student’s t-test was used to determine significance among the small groups. A value of p < 0.05 was considered to be statistically significant.

3. Results and discussion

The strategy for the synthesis of cost-effective yet efficacious macromolecular antimicrobials disclosed herein employs a polyaddition polymerization from commercially available monomers without the need for a catalyst (Scheme 1). The wide range of commercially available α,ω-tetramethyldiamines allows for polymers with a variety of hydrophobicities to be synthesized, an important parameter that is known to influence the antimicrobial activity of a polymer. Dimethylformamide (DMF) was used as the polymerization solvent as it dissolves the reactants at elevated temperatures. Since we have shown that the antimicrobial activity and molecular weights of polymers are independent of each other, we employed conditions that would suppress polymerization by diffusion limited mechanisms (i.e., polymer precipitation) to attain only modest molecular weights, which should aid in the solubility of the polymer in aqueous media. In a representative case, equimolar amounts of p-xylene dichloride and bis[2-(N,N-dimethylamino)ethyl] ether were dissolved in DMF, slowly combined under nitrogen, and stirred at 85 °C overnight to obtain a precipitate. The precipitate was filtered and washed with diethyl ether to afford polymer 4 as a hygroscopic solid with number averaged molecular weight (Mₙ) = 7930 Da and polydispersity index (D) = 2.6, as determined by gel permeation chromatography (GPC) [Fig. 1a]. The 1H NMR spectrum of polymer 4 in D₂O exhibited signals that are consistent with the formation of quaternary ammonium centers, characterized by downfield shifts of the N-methyl (from 2.15 to 3.10 ppm) and N-methylene (from 2.48 to 4.07 ppm) proton signals of the bis[2-(N,N-dimethylamino)ethyl] ether segment of the polymer relative to that of the starting material [Fig. 1b]. With this methodology, we synthesized a library of aryl groups-containing antimicrobial polymers (Scheme 1). As shown in Table 1, under these reaction conditions, the weight averaged molecular weights...
(M_w) of the polymers remained below 10000 Da, with the exception of polymer 4 due to its exceptional solubility in DMF. It was noted that the polymers possess a wide molecular weight distribution (Table 1). As hydrophobicity of polymers plays a key role in the antimicrobial function of polymers, water-octanol partition coefficient (log P) of polymers was therefore measured to investigate the impact of overall polymer hydrophobicity on antimicrobial activity (Table 2). As expected, the introduction of positive charges along the polymer backbone rendered these materials water-soluble, although limited water solubility was observed for polymers 2 and 3, presumably due to their more rigid structure and higher hydrophobicity as compared to polymer 1 or polymer 4 (log P: −1.90, −0.94 and −3.20 for polymers 1, 2 and 4, respectively; Log P of polymer 3 was not measured as it is not water soluble).

Assays against clinically relevant microbes: Gram-positive bacteria S. aureus, Gram-negative bacteria E. coli and P. aeruginosa, and fungi C. Albicans were performed to ascertain the antimicrobial activity of the polymers. The broth microdilution method was used to determine the MICs against these microbes, which are the lowest polymer concentrations at which microbial growth was completely inhibited after treatment. As shown in Table 2, polymers 1 and 4 demonstrated more potent antimicrobial activity, with extremely low MICs of 3.9–7.8 µg/mL, than polymer 2. It is noteworthy that polymers 1 and 4 exhibited high potency towards Gram-negative bacteria and inhibited the growth of E. coli and P. aeruginosa at 7.8 and 3.9 µg/mL, respectively. This result is of great importance, as

Fig. 1. Physical characterizations of polymer 4. (a) GPC trace (mobile phase 54/23/23 (v/v/v%) water/methanol/acetic acid and 0.5 M sodium acetate); (b) 1H NMR spectrum (D2O). * = H2O.
there are limited antibiotics available for treating Gram-negative bacteria. In particular, P. aeruginosa are resistant to most antibiotics. The remarkable antimicrobial activity observed for polymers 1 and 4 might be due to the fact that they adopt alternating amphiphilic structures, where the cationic hydrophilic and hydrophobic groups are alternatively distributed in the polymer chain. This conformation is highly dynamic, and offers excellent accessibility of the hydrophobic components upon contact with the negatively charged microbial surface. The hydrophobic residues hence facilitate their insertion into the lipid bilayer and disruption of the cytoplasmic membrane, leading to microbial lysis. Similar findings were reported in our previous study, wherein poly(carbonates with randomly distributed hydrophobic repeat units displayed enhanced antimicrobial activity as compared to the corresponding block copolymer analog due to better accessibility of the hydrophobic components [43]. However, the latter self-assembled into stable micelles and shielded its hydrophobic portions within the micellar core, and were thereby excluded from interacting with the bacterial cell membrane, making them ineffective against Gram-negative bacteria. In addition to hydrophobicity, the aromatic functionality may be another critical parameter for the interaction between phospholipid bilayer and polymer during penetration [44,45]. Polymer 2 with stronger hydrophobicity exhibited lower antimicrobial activity as compared to polymers 1 and 4, likely because polymer 2 aggregated in the bacterial culture medium due to its limited water solubility.

Having observed promising broad-spectrum antimicrobial activity for polymers 1 and 4, we then sought to further enhance antimicrobial properties by systematically bolstering the hydrophobic content. Thus, p-xylene dichloride was copolymerized with varying ratios of TMEDA and N,N,N’,N’-tetramethyl-p-phenylenediamine as shown in Scheme 1, essentially forming statistical copolymers of similar molecular weights comprising the components of polymers 1 and 2 (Table 1, entries 5–7, polymers 1a–c). Similarly, copolymerization of the dichloride with bis[2-(N,N-dimethylamino)ethyl] ether and N,N,N’,N’-tetramethylbenzidine afforded copolymers resembling combinations of polymers 3 and 4 (Table 1, entries 8–10, polymer 4a–c). As shown in Table 2, we observed enhanced antimicrobial activity for polymer 1a as compared to polymer 1 due to increased hydrophobicity (log P: −1.90 and −1.71 for polymers 1 and 1a, respectively). This is because the increased hydrophobicity enhanced the interactions with the lipid domains of microbial membrane, disrupting the membrane more effectively. This finding is consistent with our previous observations that an increased content of hydrophobic monomer led to a reduced MIC [36,46]. However, further increasing the content of N,N,N’,N’-tetramethylbenzidine to 40% (polymer 1b) and 60% (polymer 1c) did not affect antimicrobial activity (log P: −1.68 and −1.60, respectively). Similar observations were also reported by Kuroda et al., where antimicrobial activity of polymers increased with increased butyl methacrylate composition up to 30%, and further increase of the butyl methacrylate composition did not affect the MIC [21]. This might be attributed to the fact that further increase in hydrophobicity might induce polymer aggregation in the bacterial culture medium, preventing antimicrobial action. Similarly, polymers 4a and 4b with increased hydrophobicity (log P: −1.85 and −1.73, respectively) exhibited an increased potency in antimicrobial activity especially against S. aureus and P. aeruginosa as compared to polymer 4 (log P: −3.20) (Table 2). Furthermore, bis[2-(N,N-dimethylamino)ethyl] ether was copolymerized with varying ratios of p-xylene dichloride and 4,4’-bis(chloromethyl)-1,1’-biphenyl as shown in Scheme 1. Polymer 6 with higher hydrophobicity (log P: −0.65) exhibited a lower antimicrobial effect than polymer 5 (log P: −1.35) possibly due to aggregation in the bacterial culture medium. Nonetheless, both polymers exhibited strong antimicrobial activity against all the microbes tested.

The antimicrobial activity of triclosan and chlorhexidine digluconate, which are widely used in personal care products, was compared with the polyionenes under the same testing conditions. Both of them exhibited a lower antimicrobial effect especially against Gram-negative bacteria than the polymers (Table 2). Compared to other polymers, polymer 1 displayed excellent antimicrobial potency and the highest selectivity (HC50/MIC > 732, where MIC was defined as the average MIC of polymers against the various microbes) (Table 2), and was therefore selected for further studies.

### Table 1

<table>
<thead>
<tr>
<th>Polymer</th>
<th>M&lt;sub&gt;n&lt;/sub&gt; (GPC)</th>
<th>M&lt;sub&gt;w&lt;/sub&gt; (GPC)</th>
<th>M&lt;sub&gt;n&lt;/sub&gt;/M&lt;sub&gt;w&lt;/sub&gt;</th>
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<tbody>
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<tr>
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<tr>
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<tr>
<td>1f</td>
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<td>1.91</td>
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</table>

<sup>a</sup> Insoluble in GPC solvent.

### Table 2

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MIC (µg/mL)</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>C. albicans</th>
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<tr>
<td></td>
<td>S. aureus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>Chlorhexidine digluconate</td>
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<table>
<thead>
<tr>
<th>Polymer</th>
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<th>Log P</th>
<th>Selectivity&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
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<td>5</td>
<td>800</td>
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<tr>
<td>6</td>
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<tr>
<td>Chlorhexidine digluconate</td>
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<tr>
<td>Triclosan</td>
<td>N.T</td>
<td>N.T</td>
<td>N.T</td>
</tr>
</tbody>
</table>

<sup>a</sup> Insoluble in GPC solvent.

<sup>b</sup> Selectivity is calculated as HC50/MIC, where MIC was defined as the average MIC of polymers against various microbes.
To assess the activity of polymers under physiological conditions, the MIC values of polymer 1 were measured in the presence of 10% FBS. The MICs against *E. coli* and *P. aeruginosa* slightly increased to 15.6 μg/mL, indicating that polymer 1 retained its potency even in the presence of serum. This is of importance for its application under physiological conditions as peptides and antibiotics were reported to have reduced antimicrobial activity in the presence of serum due to association with serum proteins [47].

To determine the microbial properties of the polymers, colony counting assays were performed. Bacterial samples (*S. aureus* and *E. coli*) treated with polymer 1 at MIC and 2 × MIC for 18 h showed that the bacteria were completely eradicated (no colonies were observed), indicating a microbicidal mechanism. For clinical applications, the disinfec ting agents should exert potency in a rapid bactericidal manner. The killing kinetics of the polymers was further evaluated on *S. aureus* and *E. coli* using polymer 1 as an example. As illustrated in Fig. 2a, the polymer caused a greater than 2-log reduction in the number of viable *S. aureus* colonies (>99% killing efficiency) within 2 min, and eradicated all the bacterial cells in 10 min at 4 × MIC (i.e. 15.6 μg/mL). At 31.3 μg/mL, a killing efficiency of ~100% was obtained in 15 s. In the case of *E. coli*, the killing efficiency was almost 100% with concentrations of 15.6 μg/mL and 31.3 μg/mL (Fig. 2b) at 2 min. For *P. aeruginosa*, which is notoriously difficult to kill, the killing efficiency was 99.5% (>2-log reduction of the initial inoculum) and ~100% with a concentration of 100 mg/L at 10 min and 20 min, respectively (Fig. 2c). Notably, *P. aeruginosa* were completely killed by polymer 1 in less than 2 min at a higher concentration of 200 μg/mL (Fig. 2c). To the best of our knowledge, polymer 1 possesses the fastest biocidal kinetics over a broad spectrum of microbes compared to the numerous antimicrobial peptides and polymers published in the literature thus far.

The antimicrobial activity of polymers was further investigated using clinically isolated multidrug-resistant bacteria and fungi. As seen in Table 3, polymer 1 effectively inhibited the growth of all microbes with low MIC values of 1.95–31.3 μg/mL, demonstrating strong and broad-spectrum antimicrobial activity against multidrug-resistant bacteria *S. aureus*, MRSA, *E. coli*, *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, as well as fungi *C. albicans* and *C. neoformans*. It is noted that treatment of clinically isolated *E. coli* and *P. aeruginosa* with polymer 1 showed slightly higher MIC value as compared to that in the ATCC strain (15.6 vs 7.8 μg/mL). This was possibly due to the fact that the phospholipid composition of cell membranes differed with different strains.

To assess the potential emergence of bacterial resistance after repeated use of cationic polymers, bacteria were exposed multiple times to polymer 1 at a sub-lethal concentration (1/8 of MIC). The conventional antibiotic ciprofloxacin was used as the control. Multiple treatments with ciprofloxacin induced drug resistance at passage 4, as evidenced from the doubling of the MIC value (MIC0/MIC = 2, MIC0 = 3.9 μg/mL). This was followed by a 4-fold increase in MIC value at passage 7 (Fig. 3), indicating the emergence of resistant strains. There were a number of studies reporting chlorhexidine-resistant *S. aureus* isolated from patients, and chlorhexidine-resistant methicillin-resistant *S. aureus* (MRSA) [48,49]. More recently, reduced levels of microbial susceptibility to chlorhexidine were reported [50]. Notably, repeated treatments with polymer 1 did not induce any drug resistance during the course of study over 10 passages.

To elucidate the antimicrobial mechanism of the polymers, we investigated the membrane permeability changes before and after incubation with polymer 1 for 2 h at 2 × MIC and 4 × MIC using propidium iodide (PI) and FITC-labeled dextran dye (100 kDa). It was observed that there was no fluorescence in the untreated *S. aureus*, suggesting that the bacterial membrane was intact (Fig. 4). In sharp contrast, cells treated with polymer 1 showed red fluorescence at both concentrations, indicating that PI had intercalated with DNA upon entering the membrane-disintegrated cells via passive diffusion. Disintegration of bacterial cell membranes was also observed by uptake of FITC-labeled dextran. These observations clearly revealed that the antimicrobial property of polymer 1 was derived from the disruption of the bacterial membrane. As the size of FITC-labeled dextran particles in solution was ~13 nm or larger, the size of the pores induced by polymer 1 on the membrane of *S. aureus* was likely to be ~13 nm or larger. This finding is in agreement with the membrane lytic mechanism of various synthetic antimicrobial polymers reported in the literature [19]. In addition, after treatment at 4 × MIC, the intensity of
fluorescence was greater, indicating that polymer 1 demonstrates membrane disintegration in a dose-dependent manner. In the case of *P. aeruginosa*, strong red fluorescence for PI was seen for the treated cells, whereas weak green fluorescence for FITC-labeled dextran was observed (Fig. 4). The results revealed that polymer 1 caused a greater extent of membrane disruption towards *S. aureus* as compared to *P. aeruginosa*. This correlated well with the varying antimicrobial activity of polymer 1 towards *S. aureus* as compared to *P. aeruginosa* (Table 2, Fig. 2). It is expected that the physical disruption of bacterial cell membranes by polymer 1 will provide advantages for various medical applications due to the reduced probability of developing drug resistance.

Hemolysis has been commonly used as a preliminary assessment of selectivity of antimicrobial agents over mammalian cells. The hemolytic behavior of the polymers towards rat red blood cells (RBCs) was evaluated at various concentrations. As shown in Table 2 and Fig. 5a, negligible or no hemolysis was observed for all polymers at their respective MIC values. Notably, polymer 1 showed negligible hemolytic activity even up to 5000 µg/mL (the highest concentration tested, Fig. 5b), demonstrating its excellent selectivity toward a broad range of pathogenic microbes over mammalian cells. This also indicates that polymer 1 binds weakly to the RBCs membrane, and there was no polymer permeation into RBCs membrane. Hydrophobic/hydrophilic balance of an antimicrobial polymer is a pivotal structural determinant of how the polymer interacts with cellular membranes, and as a result impacts selectivity of microbes over mammalian cells. The log P values of the polymers were thus used to better understand the impact of overall polymer hydrophobicity on its biological activity. The hemolytic activity (HC50 values) increased with an increase in the hydrophobicity of cationic polymers. For instance, the log P value of polymer 2 bearing aromatic substitutions of higher hydrophobicity was significantly higher than that of polymer 1 (log P: −0.94 and −1.90, respectively). As such, polymer 2 was far more hemolytic (HC50 = 188 µg/mL) than polymer 1 (HC50 > 5000 µg/mL). Polymer 4 containing bis[2-(N,N-dimethylamino)ethyl] ether substituents of lower hydrophobicity (log P = −3.2) when compared to polymer 1 caused 20% hemolysis at 1000 µg/mL because of its higher molecular weight. Compared to polymer 1, polymers 1a–c of greater hydrophobicity (log P: −1.71, −1.68 and −1.60, respectively) were more hemolytic (HC50: 1000, 500 and 500 µg/mL, respectively). Similarly, hemolytic activity of polymers 4–6 increased with increasing hydrophobicity (log P: −3.2, −1.35 and −0.65, respectively). This observation is consistent with previous reports that increased hydrophobicity enhances hemolytic activity due to strong interaction between the polymer and the lipid region of the cellular membrane [21]. The results suggest that by rationally designing cationic group structure, it is possible to yield polymers with remarkable selectivity towards microbial cells and low toxicity to mammalian cells. For potential application as topical antimicrobials and antiseptics, the cytotoxicity of polymer 1 was evaluated in human dermal fibroblasts (HDFs). Even with 500 µg/mL of polymer 1, the cell viability was more than 80% after 2 min of treatment (Fig. 5b). The cytotoxicity of polymer 1 was further investigated by determining the amount of LDH enzyme released by HDFs with damaged membranes (Fig. 5c and d). Interestingly, the LDH release (cytotoxicity) in HDFs exposed to polymer 1 for 2 min was less than 2% at concentrations of 4–500 µg/mL (Fig. 5c). However, HDFs exposed to chlorhexidine digluconate for 2 min demonstrated significantly higher LDH release than polymer 1 when the concentration was above 125 µg/mL. Even when the treatment time with polymer 1 increased to 1 h, LDH release in HDFs was still less than 10% at concentrations up to 500 µg/mL (Fig. 5d). In sharp contrast, increasing the concentration of chlorhexidine digluconate and its exposure time drastically increased its cytotoxicity (e.g. more than 80% at a concentration of 125 µg/mL for 1-h treatment) (Fig. 5c and d). Taken together, the results indicated that polymer 1 exhibited excellent biocompatibility with HDFs.

To demonstrate its safety for future clinical applications (e.g. surgical scrubs), polymer 1 was evaluated for dermal toxicity. Mice were randomly grouped (five in each group), and skin treated with six topical solutions twice a day over 5 days. The six treatments were as follows: water (control), polymer 1 (200 and 500 µg/mL), betadine (control), chlorhexidine (neutral, control, 4% w/v in Bacitreal handwash) and benzalkonium chloride (cationic, control, 0.198% w/v, a concentration used in Dettol handwash). These samples were evaluated specifically for any compromise in cellular integrity, changes in tissue structure or evidence of inflammation in the epidermis, dermis and subcutaneous levels. As shown in Fig. 6b and c, in the samples treated with polymer 1 (200 and 500 µg/mL), no significant accumulation of inflammatory cells was seen. Mild interstitial oedema (accumulation of fluid in dermis, arrow 2) not amounting to a pathology and mild parakeratosis (thickening of top-most layer of epidermis, arrow 1) were observed. Epidermal cellular vacuolization (intracellular oedema signifying cell death) and acantholysis (separation of adjacent cells due to loss of intercellular adhesions) that signify early cellular damage were not observed. Importantly, the overall structure and cellular integrity of the skin was preserved. In the samples treated with the positive controls, including clinically used betadine (povidone-iodine) and

Table 3
Antimicrobial activity of polymer 1 against clinically isolated microbes.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> 34698</td>
<td>3.9</td>
</tr>
<tr>
<td>MRSA 34712</td>
<td>3.9</td>
</tr>
<tr>
<td>MRSA 34689</td>
<td>7.8</td>
</tr>
<tr>
<td><em>E. coli</em> 34723</td>
<td>15.6</td>
</tr>
<tr>
<td><em>E. coli</em> 34729</td>
<td>15.6</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 25844</td>
<td>15.6</td>
</tr>
<tr>
<td><em>A. baumannii</em> 34727</td>
<td>3.9</td>
</tr>
<tr>
<td><em>A. baumannii</em> 34716</td>
<td>15.6</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> 34693</td>
<td>31.3</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> 34694</td>
<td>15.6</td>
</tr>
<tr>
<td><em>C. albicans</em> 34092</td>
<td>7.8</td>
</tr>
<tr>
<td><em>C. neoformans</em> 34709</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Fig. 3. Changes in MIC of antimicrobial agents against *E. coli* 25922 upon multiple sub-lethal dose exposures.
Fig. 4. Confocal microscopic images of (a) *S. aureus* and (b) *P. aeruginosa* incubated with polymer 1 for 2 h at various concentrations (red fluorescence: PI; green fluorescence: FITC-labeled dextran (100 KDa)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
chlorhexidine, however, varying extents of damage to the skin was noted (Fig. 6d–f). There were spongiotic changes (significant intercellular accumulation of fluid in epidermis) associated with inflammatory infiltration in the epidermal to dermal layer (arrow 3), suggesting significant dermal inflammation. In the epidermal layer, there was evidence of acantholysis (intercellular separation and rounding up of cells), denoting an early insult to the skin (arrow 5). Acantholysis surrounding the hair follicular units could also be seen (arrow 6). The findings of acanthosis (thickening of the epidermal layer, arrow 4), parakeratosis and hyperkeratosis (thickening of dead skin, the top-most layer of epidermis) also suggested a cumulative irritation effect to the skin.

The results indicated that topical application of polymer 1 (200 and 500 μg/mL) showed a better skin compatibility profile in mice as compared to the clinically used surgical scrubs betadine and chlorhexidine. Acute oral toxicity (LD50, lethal dose at which half the mice are killed) was studied to further evaluate whether the compound would be safe to handle. LD50 of polymer 1 was estimated to be 1871 mg/kg via oral administration, indicating that the polymer has low toxicity. Moreover, most surgical scrubs and hand sanitizers in hospitals and clinical environments are alcohol-based, e.g., betadine. Non-alcohol based formulations are desirable due to their non-dehydrating characteristics. They are gentler on skin particularly in the presence of cuts and chapped hands and less expensive. Except for polymers 3, all other polymers are soluble in water, easy to be formulated into non-alcohol based surgical scrubs and hand sanitizers.

To evaluate the in vivo bactericidal activity of polymer 1, P. aeruginosa was introduced to contaminate the normal dorsal skin of mice [42]. The contaminated skin was treated with polymer 1 (500 μg/mL) and the clinically used surgical scrub chlorhexidine (4% w/v in Bactishield handwash); mice without treatment were employed as control. The number of P. aeruginosa recovered from the contaminated skin treated with polymer 1 and chlorhexidine handwash for 2 min (2.50 and 3.59 log10 CFU) was found to be significantly reduced as compared to the control (5.25 log10 CFU, p < 0.0005, Fig. 7). The treatment with polymer 1 decreased the number of P. aeruginosa on the skin more effectively than chlorhexidine handwash (p < 0.01, Fig. 7).

4. Conclusion

Potent and cost-effective antimicrobial polymers have been successfully synthesized through simple addition polymerization using commercially available, relatively inexpensive starting materials. They have demonstrated strong and broad-spectrum microbicidal activity through membrane-lytic mechanism and ability of mitigating drug resistance. By varying the ratio of hydrophobic-to-hydrophilic groups, polymers with high potency and low cytotoxicity can be easily synthesized. At the optimal compositions, the antimicrobial polymer exhibits rapid killing kinetics over a broad spectrum of microbes. More importantly, the polymer shows superior skin compatibility and in vivo bactericidal activity, as compared to the clinically used surgical scrubs betadine and chlorhexidine. Thus, the polymers reported herein may be promising antimicrobial agents, e.g., as antimicrobial surgical scrubs for the prevention of MDR infections.
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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2017.02.027.

References


Cytotoxicity of polycations: Relationship of molecular weight and the hydrolytic theory of the mechanism of toxicity

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**A B S T R A C T**

The mechanism of polycation cytotoxicity and the relationship to polymer molecular weight is poorly understood. To gain an insight into this important phenomenon a range of newly synthesised uniform (near monodisperse) linear polyethyleneimines, commercially available poly(L-lysine)s and two commonly used PEI-based transfectants (broad 22 kDa linear and 25 kDa branched) were tested for their cytotoxicity against the A549 human lung carcinoma cell line. Cell membrane damage assays (LDH release) and cell viability assays (MTT) showed a strong relationship to dose and polymer molecular weight, and increasing incubation times revealed that even supposedly “non-toxic” low molecular weight polyners still damage cell membranes. The newly proposed mechanism of cell membrane damage is acid catalysed hydrolysis of lipidic phosphoester bonds, which was supported by observations of the hydrolysis of DOPC liposomes.

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1. Introduction

Polycations are materials that find application as delivery vectors in the field of DNA and RNA based therapies, because they condense polyanionic nucleic acids. This is a field with enormous promise, but one that has failed to achieve its full potential despite great clinical interest; mostly due to the toxicity of the polycationic carriers. (Behr, 2012; Gary et al., 2007; Pack et al., 2005) Further problems arise due to the fact that polymeric transfection methods require a large excess of polycations, which is not associated with the poly(nucleic acid), (Boeckle et al., 2004; Dai et al., 2011; Yue et al., 2011) and this present major limitations on in-vivo transfection due to different trafficking of the two populations.

Polyethyleneimine (PEI) is a cationic polymer which is commonly available with branched and linear structures, and has been synthesised in hairy, comb and cyclic structures. The branched structure is synthesised via the aqueous cationic polymerisation of aziridines, whilst the linear form is typically synthesised by the hydrolysis of a poly(2-oxazoline). (Monnery and Hoogenboom, 2015) Poly(L-lysine) is also commonly available via the ring-opening polymerisation of N-carbobenzoxy-L-lysine-N-carboxy anhydride. (Fasman et al., 1961) Since these polycations are some of the most frequently used in cell transfection, this work concentrates on assaying these materials.

The mechanism of cytotoxicity of polycationic materials, such as PEI, is a poorly understood matter. (Parhamifar et al., 2010) It is known that polycationic materials do not produce an apoptotic response, but rather cell death is due to necrosis, (Fischer et al., 2003) and that a variety of organelles are damaged. (Grandinetti et al., 2011; Grandinetti et al., 2012; Moghimi et al., 2005) The mechanism of the necrotic damage is not yet understood. Here we aim to investigate the effect of the properties of the polymer on cell membrane damage.

There is a significant body of evidence that polycations open pores in cellular membranes. Banaszak-Holl and coworkers have shown that a wide variety of cationic macromolecules open pores in a supported phospholipid bilayers. (Hong et al., 2004; Hong et al., 2006; Leroueil et al., 2008; Mecke et al., 2005) Poration of the cells is a common way of transfecting cells, with various physical
methods (electroporation etc.) and chemicals such as surfactants allowing polynucleic acids to simply diffuse into the cells. (Hapala, 1997) However, commonly used polycations such as PEI and PLL should not act as surfactants.

It has been shown that the presence of uncomplexed polycation is responsible for the cell damage, but without their presence there is limited transfection. (Boeckle et al., 2004; Fahrmeir et al., 2007; Hanzlikova et al., 2011; Yue et al., 2011) Kichler et al. reported that PEI has no effect on cell membranes. (Kichler et al., 2001) However, the underlying experiments were carried out in citrate buffer, and the PEI was hence sequestered into an uncharged PEI–citrate complex (Eberhardt et al., 2009), so no free polymer was present.

The effect of cationic polymer molecular weight on the cytotoxicity is poorly understood. Fischer et al. claim that higher molecular weight materials are more toxic, (Fischer et al., 2003) but this is based on a rather random selection of cationic polymers as only one molecular weight of a range of different polymers is assayed and compared. Long et al. performed a systematic survey of the toxicity of poly(N,N-dimethyldiallylamineethyl methacrylate)s, but unfortunately the lowest molecular weight assayed (43 kDa) was so toxic that no relationship can be discerned, although toxicity was clearly due to membrane lysis. (Layman et al., 2009) Two groups (Coll et al. and Wurm et al.) have independently fractionated a commercial broadly-polydispers linear PEI (1 kDa) and assayed the individual fractions for transfection efficiency. They both reported that fractions below 4 kDa show little transfection activity and little toxicity, and fractions above 20 kDa show little activity but high toxicity, with a maximum transfection efficiency at ca. 15 kDa. (Falcó et al., 2009; Kadleceva et al., 2012) However, neither group performed an LDH or similar assay to assess the effect of the molecular weight on cell membrane disruption. Boe et al. assayed a limited range of commercial PEI samples of both linear and branched structures on osteosarcoma cells, with mitochondrial activity measured by (3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) after 24 h exposure. However, the limited range of materials (i.e. no polycations between 2.5 and 25 kDa) and lack of LDH or similar assays make further interpretation difficult (Boe et al., 2008). In none of these cases the degree of cell membrane disruption has been assessed (i.e. by the lactose dehydrogenase assay), and since the toxicity is clearly due to the disruption and poration of cellular membranes this is clearly a major gap in our knowledge, which will be addressed in the current work.

The mechanism by which polycations induce pores on cellular membranes remains obscure, and essentially two reasonable models exist: either the polycations act as a surfactant (Vaidyanathan et al., 2016) or as a proton transfer catalyst. (Seddon et al., 2009) In the latter, the polycations acts as a proton transfer catalyst that could lead to the hydrolysis of the phospholipids and changes in the curved elastic stress of the membrane. This induces the formation of inverted hexagonal phases in the lipid bilayer (“pores”) and phase separation of lysophospholipids which form blebs, this being demonstrated for low molecular weight cationic materials. (Baciuc et al., 2006; Casey et al., 2014; Casey, 2011; Shearmen et al., 2007) The latter requires the cationic polymer to stabilise a pore, and thus remain localised in the pore. This has never been observed, although the surfactant effect may explain poration by amine functionalized silica or gold nanoparticles, which has been observed by Banaszak-Holl and coworkers.

The hypothesis of this work is that the toxicity of polycations increases with molecular weight (assuming the same structure and architecture), and is due to a greater degree of membrane disruption. To test this a systematical series of 1-PEI covering a broad molar mass range has been synthesised (Monnery et al., 2015) and tested for mitochondrial activity and cell membrane damage in the A549 cell line, in comparison to 25 kDa hyperbranched PEI (b-PEI) and a series of poly(lysine)s (PLL).

To address the postulated membrane disruption by either hydrolysis or surfactant mechanism, experiments were undertaken to evaluate the hydrolysis of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in presence of PEI by HPLC.

2. Materials and methods

2.1. Materials

Calcium hydride (93%), 2-ethyl-2-oxazoline (99%), methyl tosylate (98%), dimethyl sulfoxide (98%), thiazolyl blue tetrazolium bromide (98%), various poly(lysine)s, 1,2-dioleoyl-sn-glycero-3-phosphocholine, various poly-lysines and 25 kDa hyperbranched PEI were purchased from Sigma-Aldrich. Water used was purified by reverse osmosis to 18 MΩ (Barnstead Nano-pure). Hydrochloric acid (37%), sodium hydroxide (99%+, pellets), DCM (99.8%+) and diethyl ether (99.8%+) were purchased from VWR (Lutterworth, UK). Phosphate buffered saline, DMEM (Glutamax), OptiMEM and fetal calf serum were purchased from Gibco (Thermo Fisher Scientific Inc., Loughborough, UK). LDH release assay were purchased from Promega (Madison, WI, U.S.A.) under the name “CytoTox 96® Non-Radioactive Cytotoxicity Assay”.

Standard glassware was used throughout. The polymerisation vessel was an oven dried two neck flask equipped with a tap (for addition of liquid reagents and sampling) and a condenser with an isolation tap connected to the Schlenk line via rubber tubing and was flame dried under vacuum before use. Liquid reagents were handled with vacuum dried gas-tight syringes (Hamilton, Bona-
duz, Switzerland) using Schlenk technique. Glassware for hydrolysis to 1-PEI etc. was similar, although less rigorous procedures were adopted in light of the aqueous solvent.

2.2. Instrumentation and polymer characterisation

Polymers were analysed by Size Exclusion Chromatography (SEC) and Nuclear Magnetic Resonance Spectroscopy (NMR). The SEC was a Polymer LaboratoriesGPC-50 with 2 x PLGEL MIXED-D (300 × 75 mm) columns and a guard column (50 × 75 mm MIXED-D), using DMF (1% v/v) triethylamine and acetic acid); poly(methyl methacrylate) (PMMA) standards were used as calibrants. A sample of the crude reaction mixture was mixed with an equal volume of deuterated chloroform and analysed via 1H NMR spectroscopy on a Bruker DX-400 machine and the degree of conversion was determined by integrating the polymer backbone (δ = 3.1–3.8 ppm) and the two methylene peaks (4 and 5 position) of the oxazoline ring (δ ~ 3.8 and 4.1) using the equation:

\[ P = \sum (\delta(\text{backbone}) + \delta(\text{monomer}^4) + \delta(\text{monomer}^5)) \]

A multi-angle light scattering DAWN EOS (Wyatt Technologies Corporation) was placed in series between the SEC column and the refraction index detector and used to determine DLS. (Shortt, 1994)
Microplates were read on a Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, California) at the appropriate wavelength. Cell images were obtained with a Zeiss Vision KS400 system (Carl Zeiss, Göttingen, Germany). High-Performance Liquid Chromatography (HPLC) eluograms were recorded on an Agilent 1100 series with quaternary pump, diode array detector, evaporative light scattering detector (Polymer Labs PL-ELS 2100) and an incubated injector. The column was a 5 cm 5 μm C18 (Phenomenex, Macclesfield, U.K.) with the elution gradient 0 min water, 11 min acetonitrile, 12 min acetonitrile, 23 min methanol, 25 min methanol, 27 min water, 30 min water. All solvent changes were isocratic. Flow rate was 2.5 ml/min.

2.3. Synthesis of poly(2-isopropyl-2-oxazoline) and hydrolysis to l-PEI

The synthesis of these materials has been reported. (Monnery et al., 2015)

2.4. Polymerisation of EtOx500

The polymerisation was carried out essentially as per the commercial product in-vivo jetPEI. (Adib et al., 2010) Into a two neck round bottom flask with stirrer and reflux condenser was added 2-ethyl-2-oxazoline (5 mL, 4.75 g, 48 mmol), dry acetonitrile and a methyl tosylate stock solution in acetonitrile for a monomer to initiator (M:I) ratio of 50 (17.85 mg MeOTs, 95.8 μmol in 10 mL acetonitrile). The mixture was refluxed under argon for 48 h, with a sample taken at 24 h (90% conversion by 1H NMR spectroscopy) and quenched at 48 h (99.5% conversion) with a 5% sodium carbonate in water solution and left to reflux overnight before being extracted into dichloromethane. The organic phase was dried with anhydrous magnesium sulfate, filtered through paper and concentrated in vacuo before precipitating into 20 vols of diethyl ether, filtering (P2 glass frit) and drying in vacuo yielded a slightly yellow powder E1 (4.2 g, 90%). The 1H NMR spectrum was consistent with the literature. (Rivas and Anánias, 1987)

\[ \text{SEC: } M_w = 24.4 \text{ kDa, } M_m = 49.8 \text{ kDa, } M_p = 61.8 \text{ kDa, } D_w = 2.04, D_{MALLS} = 1.12 \]

2.5. Hydrolysis to l-PEI500

\[ \text{P(EtOx)}_{500} (1.40 \text{ g, } 14 \text{ mmol of repeat units}) \text{ was dissolved in } 6 \text{N hydrochloric acid (50 mL) and refluxed overnight at } 120 \degree \text{C} \text{ in an oil bath. The volatiles were removed in vacuo, the residue was dissolved in boiling water (50 mL) and made basic with sodium hydroxide (~1 g). On cooling the hydride of L-PEI precipitated, was filtered (P2 glass frit), washed with water (~1 L) until the filtrate was neutral and dried in vacuo overnight to yield a white powder. A sample (43.5 mg, 1 mmol) was placed in an HPLC vial and dissolved in propanoic anhydride (0.64 mL, 5 mmol, 5 equiv), sealed and heated to 65 °C overnight, cooled, quenched with water (1 mL) and the volatiles removed. The residue was analysed by SEC and showed no significant change in molecular weight distribution compared to the starting polymer, showing main chain stability during hydrolysis. The remainder of the product was dissolved in boiling water (1 mL) and 37% hydrochloric acid (1 mL) was added. The volatiles were removed in vacuo to yield PEI500 as a white powder (0.97 g, 86%). The \textsuperscript{1}H NMR spectrum was consistent with the literature and showed 99.6% hydrolysis. (Adib et al., 2010; Saegusa et al., 1972) \]

\[ \text{1H NMR (400 MHz, D}_{2}\text{O): } \delta = 3.5 \text{ (br, 4H, -CH}_{2}\text{-CH}_{2}\text{-NH}_2\text{Cl-)} \]

\[ \text{13C NMR (101 MHz, D}_{2}\text{O): } \delta = 44.0 \text{ (-CH}_{2}\text{-CH}_{2}\text{-NH}_2\text{Cl-)}. \]

2.6. Branched PEI hydrochloride

Freebase hyperbranched PEI (0.64 g) was dissolved in 6N hydrochloric acid (25 mL) and stirred for 1 h. Volatiles were removed in vacuo to yield 1.12 g (100%) of slightly yellow crystals. \[ \text{1H NMR (400 MHz, D}_{2}\text{O): } \delta = 2.7-3.9 \text{ (br m, 4H, -CH}_{2}\text{-CH}_{2}\text{-NH}_2\text{Cl-)}, 7.4-8.3 \text{ (br m, various amines)} \]

\[ \text{13C NMR (101 MHz, D}_{2}\text{O): } \delta = 35.5 \text{ (methylene adjacent to 1° amine in } 1\text{–}3\text{ °dial), 37.0 (methylene adjacent to 1° amine in } 1\text{–}2\text{ °dial), 43.5 (methylene adjacent to 2° amine in } 2\text{–}3\text{ °dial), 44.5 (methylene adjacent to 2° amine in } 1\text{–}2\text{ °dial), 46.5 (methylene adjacent to 3° amine in } 2\text{–}3\text{ °dial), 50.0 (methylene adjacent to 3° amine in } 1\text{–}3\text{ °dial)} \]

2.7. Investigation in the effect of molecular weight on cell membrane viability

A549 cells (European Collection of Cell Cultures) were cultured in supplemented DMEM (10% v/v FCS, 1% 5000 units/mL Penicillin/5000 units/mL Streptomycin). Subculturing and the preparation of plates are described in the supplementary information. Polycation stock solutions were made at 1 mg/mL in serum free OptiMEM (~25 mL) and filter sterilised (0.22 μm polysulphonate syringe filter), discarding the first 5 mL of filtrate (to remove filter adsorption effects). After equilibrating at room temperature for at least 24 h (to stabilise pH) serial dilutions were made with OptiMEM for final polycations concentrations of 1000 μg/mL, 100 μg/mL, 10 μg/mL and 1 μg/mL. The media in the experimental wells was carefully removed by hypodermic syringe and replaced with 100 μL of serum free OptiMEM. Once all wells had undergone the media change the process was repeated, adding the appropriate polycation solution. Wells were left for negative control (no cells), negative control (lysed cells), positive control (no polycation, vehicle control) and dilution control (no cells, but lysis buffer added). The plates were then returned to the incubator. One h before experimental readout 10 μL of Lactate Dehydrogenase (LDH) lysis buffer (1.1% triton x-100) was added to the positive control and dilution control wells. This plate is designed plate A.

The procedure for LDH/MTT experiments was thus; after the required incubation time (generally 4 h) the supernatant was carefully removed from each well of plate A with a multichannel pipette set at 150 μL and transferred to a 96 well v-bottom plate (plate B). The media of plate A was replaced with 100 μL complete media, 20 μL of MTT solution (5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in PBS) was added and the plate returned to the incubator for 5 h. Plate B was sealed with paraffin and centrifuged (245g, 4 min), then 50 μL of the supernatant was transferred to a 96 well flat bottom plate (plate C). 50 μL of reconstituted LDH was added to all wells and the plate wrapped in aluminium foil and placed in the dark. After exactly 30 min 50 μL stop solution (1 M acetic acid) was added to each well. The absorbance at 490 nm was read on an ELISA plate reader. Excess LDH release was calculated from plate B as follows:

\[ \text{LDH Release} = \frac{A_{490(\text{experimental})} - A_{490(\text{negative})}}{A_{490(\text{positive})} - A_{490(\text{negative})}} \]
Where $A_{490}$ = absorbance at 490 nm, (experimental) is the observed result, (negative) is the average result of 6 vehicle control wells, and (positive) is the positive average result of 6 control wells lysed with 1.1% Triton X-100 1 h prior to the addition of the assay reagent.

For MTT plate A was centrifuged (241g, 4 min), the supernatant removed carefully, DMSO (100 μL) was added and the plate returned to the incubator. After 30 min the absorbance at 570 nm read on an ELISA plate reader. Viability was calculated from plate A as follows:

$$\text{Mitochondrial Activity} = \frac{A_{570\text{(experimental)}} - A_{570\text{(negative)}}}{A_{570\text{(positive)}} - A_{570\text{(negative)}}}$$

Where absorbance values are analogous to those in the LDH assay.

Some MTT assays were conducted without LDH, and were performed as above without the LDH component.

$I_{20}$ and $I_{30}$ values were computed by fitting a linear model to the measurements and the log-doses for each polymer at each time-point. This linear model was used to predict the doses needed to get 50% and 20% toxicity, along with the 95% upper and lower bounds for these values determined using the LINEST function of Microsoft Excel to determine $\Delta y$.

2.8. Studies of phospholipid hydrolysis

DOPC liposomes were formed by placing 200 μL of a 10 mg/mL DOPC in chloroform solution into a 5 mL round-bottom flask, and removing the volatiles in vacuo. The DOPC was re-suspended in water and sonicated for 1 h at 40 °C to yield liposomes. To 0.5 mL of a 2 mg/mL DOPC liposome suspension as added 0.5 mL of 2 mg/mL PEI358 (both in water and prewarmed to 37 °C). The mixture was incubated at 37 °C in a temperature controlled HPLC sample chamber and samples were taken every 32 min (or 4 h) by the HPLC autosampler.

3. Results and discussion

3.1. Selection and synthesis of polymeric materials

3.1.1. Synthesis and characterisation of l-PEI

The PEI500 sample was synthesised as per an existing patent (Adib et al., 2010), so as to give a material comparable with the commercial material. It was of interest whether the fairly broad molecular weight distribution observed for the precursor poly(2-ethyl-2-oxazoline) would be preserved in the synthesised l-PEI. Hence re-acylation of the PEI freebase was conducted, and the molecular weight distribution was indeed preserved (see Fig. S1), which was consistent with a recent report. (de la Rosa et al., 2014) This is at odds with the usually proposed mechanism of chain transfer, which postulates that branch points will be hydrolysed. (Litt et al., 1975; Warakomski and Thill, 1990)

Broad l-PEI500 was synthesised by hydrolysis in 16.5% (v/v) hydrochloric acid (degree of hydrolysis (99.6%), the volatiles removed, re-dissolved in boiling water (ca. 5 mL) and made basic with NaOH. The freebase PEI crystallised as the temperature fell below ~60 °C and the PEI was washed with copious water until the filtrate was neutral. (Lambermont-Thijs et al., 2009) Each freebase PEI was converted back into the hydrochloride salt by dissolving in 1 mL of boiling water followed by the addition of 1 mL conc. hydrochloric acid, and followed by the removal in vacuo of volatiles.

The dispersity value reported in the patent literature was obtained via multi-angle light scattering. The SEC system employed here was equipped with such a device, and the observed $D_n/D_w$ was 1.12 (see Fig. S2), which is comparable to the commercial material. (Adib et al., 2010; Shortt, 1994)

The synthesis of the five narrow l-PEI polymers has been previously described. (Monnery et al., 2015)

3.1.2. Comparator polycations

Comparisons between different polycations are complicated by the fact that the common nomenclature used for PEI and PLL are different. PEI molecular weights are reported as the freebase (despite the linear form being invariably used as a hydrochloride salt), whilst PLL molecular weights are reported including the counter-ion (invariably a hydrogen bromide salt). For this reason in this paper we have decided to refer to the polymers by their $D_p$, thereby avoiding this confusion.

Branched 25 kDa PEI (b-PEI) and a range of commercially available PLL with similar molecular weights and were also chosen as comparators. The b-PEI was chosen due to its common use in gene therapy studies, and hydrochloride salt as well as the freebase was investigated. The PLL was chosen as a commonly used transfectant available in a range of molecular weights. However due to the different $pK_a$ values, the protonation states of the polymers will be different.

The $pK_a$ of linear PEI is 7.2–7.9 (Brisault et al., 2003). For branched PEI von Harpe et al. have measured the 25 kDa branched PEI itself and report a $pK_a$ of 8.4. (Suh et al., 1994; von Harpe et al., 2000) The $pK_a$ of PLL is that of lysine (10.5) and the $pK_a$ is not dispersed, unlike PEI. (Frey and Corn, 1996) This means that using the Henderson-Hasselbalch equation it can be determined that at $pH = 7.4$ approximately 55% of linear PEI secondary amines are protonated, ~90% of branched PEI amines and effectively all the PLL amines are protonated. The total number of charges per polymer can thus be determined by multiplying through by the $D_p$, which correlates with the observation that branched PEI

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure*</th>
<th>$M_w$</th>
<th>$D_P$</th>
<th>$pK_a$</th>
<th># * charges/polymer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI34</td>
<td>narrow linear PEL.HCl</td>
<td>1.3</td>
<td>34</td>
<td>7.45</td>
<td>19</td>
</tr>
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<td>2.8</td>
<td>65</td>
<td>7.45</td>
<td>36</td>
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<td>PEI136</td>
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<td>5.9</td>
<td>136</td>
<td>7.45</td>
<td>75</td>
</tr>
<tr>
<td>PEI222</td>
<td>narrow linear PEL.HCl</td>
<td>9.8</td>
<td>222</td>
<td>7.45</td>
<td>122</td>
</tr>
<tr>
<td>PEI358</td>
<td>narrow linear PEL.HCl</td>
<td>15.9</td>
<td>358</td>
<td>7.45</td>
<td>197</td>
</tr>
<tr>
<td>PEI500</td>
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<td>22.0</td>
<td>500</td>
<td>7.45</td>
<td>275</td>
</tr>
<tr>
<td>PLL4</td>
<td>linear PLL.HBr</td>
<td>0.84</td>
<td>4</td>
<td>10.5</td>
<td>4</td>
</tr>
<tr>
<td>PLL71</td>
<td>linear PLL.HBr</td>
<td>15.04</td>
<td>71</td>
<td>10.5</td>
<td>71</td>
</tr>
<tr>
<td>PLL115</td>
<td>linear PLL.HBr</td>
<td>24.04</td>
<td>115</td>
<td>10.5</td>
<td>115</td>
</tr>
<tr>
<td>PLL327</td>
<td>linear PLL.HBr</td>
<td>68.6*</td>
<td>327</td>
<td>10.5</td>
<td>327</td>
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<tr>
<td>b-PEI</td>
<td>broad hyperbranched PEI freebase</td>
<td>25.0*</td>
<td>580</td>
<td>8.4</td>
<td>522</td>
</tr>
<tr>
<td>b-PEI.HCl</td>
<td>broad hyperbranched PEI.HCl</td>
<td>25.0*</td>
<td>580</td>
<td>8.4</td>
<td>522</td>
</tr>
</tbody>
</table>

Table 1

Characteristics of polycations used in this study. Mass-average molecular weight ($M_w$) in kDa, a, ultra narrow PEI $D < 1.02$, broad PEI $D = 1.12$, PLL $D broad (P ca. 12)$, b – literature values (Brisault et al., 2003; Suh et al., 1994; von Harpe et al., 2000), c – number of cationic charges per polymer at $pH = 7.4$, determined from $pK_a$ and $D_p$, d – suppliers values.
complexes DNA to a greater extent than linear PEI, due to the higher charge density of b-PEI. (Itaka et al., 2004) The number of protonated amines per polymer is shown in Table 1.

3.2. Effect of polycations’ molecular weight on membrane integrity and cell viability

To determine whether polycations induced cell membrane disruption an LDH release assay was employed, which since it uses the supernatant only was multiplexed with an MTT assay by washing the cells in the culture well and supplying fresh media (see experimental 3.3). This assay measures the quantity of a cytosolic enzyme released from the cell, which is proportional to cell membrane disruption. This assay therefore reports the formation of permanent pores, but not transitory ones. LDH release experiments in previous reports showed qualitatively that polycations induce cell membrane damage. (Fischer et al., 2003; Ryser, 1967) By using well defined polymers of different molecular weights it will be possible to detect trends and thus provide quantitative data. The polymers were assayed by LDH release. After a 4 h exposure of the cells to the polycations the LDH release was assessed and the results are shown in Fig. 1.

Due to the relatively low toxicity of the smallest PEI cells were incubated with polymers for the 48 h and an LDH assay performed (Fig. 2). This exposure showed high levels of LDH release for all polycations at the 10 μg/mL concentration except PEI34 and PLL4. There is a drop in LDH “release” at the highest concentration due potentially to degradation of the released LDH. The data thus indicates that long-term exposure of cells with any PEI or PLL of significant molecular weight would be toxic.

To compare the toxicity data, the 4 h results were converted to semi-logarithmic dose-response curves and the line of best fit was converted into an IC20 and IC50 value by substitution of a linear data fit with 95% confidence intervals (Table 2). In the case of the low MW l-PEI it was concluded that IC20 and IC50 were much greater than 1000 μg/mL.

PLL has been previously shown to lyse cell membranes(Hill et al., 1999) and has been reported to be more disruptive than l-PEI (Oku et al., 1986). Our current results confirmed this observation. Comparing, for example, PEI358 and PLL327, which have similar number of repeat units, for the 4 h exposure experiment the PEI358 showed an IC20 4.6 times greater to PLL327, and an IC50 for the PEI that was above our measured range, whereas the IC50 of the PLL was low. This indicated that the order of toxicity was PLL > b-PEI > l-PEI. This is in agreement with the charge density of the polymers, with the most charge dense polymers being the most toxic.

Mitochondrial activity was assayed by the MTT assay using A549 lung cancer cells. Multiple exposure times (4, 24 and 48 h) of the various polymers were undertaken (multiplexed with LDH) and the cells seeded at 10,000 cells/well 16 h before experiment. At the lower exposure times there was consistently greater mitochondrial activity in all experimental wells than the negative control well, except when extremely high concentrations were used, which led to immediate cell death. This is an observed phenomenon, but is rarely commented upon. Problems with increased mitochondrial activity during MTT are known(Stockert et al., 2012), and Parhamifar et al. suggest extreme caution is required when using MTT for just this reason(Parhamifar et al., 2010). A common explanation is mitochondrial uncoupling due to pore formation, but evidence relies on isolated mitochondria, and not whole cells(Grandinetti et al., 2011; Larsen et al., 2012). A simpler explanation could be that since polycations damage cell membranes, the cell is simply attempting to maintain homeostasis after sub-lethal damage, by upregulating respiration to provide energy to repair the damage.

With 48 h incubation with polycations (seeded at 10,000 cells/well) the MTT assay showed the results presented in Fig. 3a. Experimental groups can be divided into a few that have suffered acute necrotic toxicity (the highest doses of the high MW polymers observed to disrupt cell membranes), and the majority which show mitochondrial activity values in excess of 100%, with the least toxic (by LDH) polymers showing an inverse relationship to that expected – higher exposure concentrations provoked greater mitochondrial activity. The trend continued at 24 and 48 h (Figs. 3b and 3c), although it was less pronounced and at 48 h exposure with few experimental groups having greater than 100% mitochondrial activity. IC20/IC50 values are estimated in Table 3 in analogous fashion to Table 2.

Fig. 1. LDH release assay on A549 cells after 4 h exposure. n = 4 ± SD. Polymers information is provided at Table 1.
The 4 h exposure of cells to polycations and the assessed LDH and MTT are plotted against each in Fig. S4. Ideally, if the two assays are complementary there should be a correlation. In our case this was indeed observed, showing complementarity of the two assays (Fig. S4). This suggests that the major cause of toxicity is the disruption of cellular membranes. The fact that mitochondrial activity is preserved even with very significant (ca. 30%) LDH release shows that the cells can survive with major cell membrane damage when exposure to polycations is for short periods of time.

Cells were microscopically investigated, and micrographs of cells exposed to polycations for 48 h at 10 μg/mL are shown in Fig. 4. The control wells (Fig. 4a) show the typical morphology of A549 cells. With exposure to low MW polycations (PEI65) some squibs and cell debris are visible, but cells show generally healthy morphologies (Fig. 4b). As MW of polycation increases e.g. for PEI136 treatments the cells become rounded and lysed cells are visible (Fig. 4c), further increases to PEI222 shows the majority of cells dead (Fig. 4d), and at higher MW (PEI500) the vast majority of cells are lysed (Fig. 4e). PLL shows the same pattern (PLL327 shown in Fig. 4f). The same squib like cell debris was observed within 30 min by Martinez et al. with 10 mM poly(allylamine hydrochloride). (Martinez et al. 2011). Since observations of these squibs have been made with PEI (both linear and branched), PLL and PAA it seems likely that this is a general feature of polycationic disruption of cell membranes. These squibs have also recently been observed for a variety of low molecular weight cationic amphiphiles (Casey, 2011).

This data shows that even relatively small polycations, such as PEI65 and PLL71 can induce cytotoxicity in the long term, even if minimal toxicity is observed during a 4 h exposure (typical for a transfection experiment). Only the extremely low molecular weight materials (PEI34, PLL4 and PLL71) were well tolerated for 48 h, producing less than 25% LDH release, although excess LDH release was still observed (Fig. 2), and an apparent increase in mitochondrial activity (Fig. 3c) showed that the cells were being stressed. Some cell debris was visible with PEI34 indicating that the membrane was being disrupted, but the cells were able to repair themselves.

### Table 2

<table>
<thead>
<tr>
<th>Material</th>
<th>LDH 4h</th>
<th>LDH 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (95% CI)</td>
<td>IC50 (95% CI)</td>
</tr>
<tr>
<td></td>
<td>IC20 (95% CI)</td>
<td>IC20 (95% CI)</td>
</tr>
<tr>
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<td>&gt;&gt; 1000</td>
<td>173.5 (83.7–359.6)</td>
</tr>
<tr>
<td>PEI65</td>
<td>&gt;&gt; 1000</td>
<td>2.6 (2.3–2.9)</td>
</tr>
<tr>
<td>PEI136</td>
<td>1209 (1085–1347)</td>
<td>4.1 (1.5–10.9)</td>
</tr>
<tr>
<td>PEI222</td>
<td>23.3 (18.3–29.5)</td>
<td>3.4 (12–9.5)</td>
</tr>
<tr>
<td>PEI500</td>
<td>12.2 (6.5–22.7)</td>
<td>3.1 (1.2–8.1)</td>
</tr>
<tr>
<td>PLL4</td>
<td>&gt;&gt; 1000</td>
<td>3.3 (1.3–6.6)</td>
</tr>
<tr>
<td>PLL71</td>
<td>65.8 (21.8–198.4)</td>
<td>2.9 (1.2–12.9)</td>
</tr>
<tr>
<td>PLL115</td>
<td>13.0 (10.3–16.6)</td>
<td>2.9 (1.2–12.9)</td>
</tr>
<tr>
<td>PLL327</td>
<td>4.5 (2.9–7.0)</td>
<td>3.9 (1.2–12.9)</td>
</tr>
<tr>
<td>25b-PEI</td>
<td>4.1 (2.4–7.0)</td>
<td>6.2 (2.9–13.3)</td>
</tr>
<tr>
<td>25b-PEI.HCl</td>
<td>6.3 (4.2–9.5)</td>
<td>28.9 (16.9–49.5)</td>
</tr>
</tbody>
</table>

**Fig. 2.** LDH release assay on A549 cells after 48 h exposure, n = 4 ± SD.
3.3. Hydrolysis of phospholipids by l-PEI

A study was conducted to assess Baciu et al.’s claim that PEI hydrolyses phospholipids, with the acidic proton being the protonated amine (Baciu et al., 2006). Control experiments showed no hydrolysis of DOPC by pure water at 37 °C over a week. At 37 °C PEI358 (which should be optimal for transfection) was mixed with DOPC at a ratio of 9.9 amines per DOPC (calculated to be the approximate ratio in a tissue culture well during transfection experiments). Hydrolysis was rapid, and apparently zero-order with respect to both reactants, and at the four h time point almost half of the DOPC had been hydrolysed (Fig. 5). Initial experiments incubating a wide range of different MW PEI in identical conditions (Fig. S5) suggest a molecular weight effect, which will be the subject of further investigation.

3.4. Suggested mechanism of poration

There is a strong molar mass effect on the destruction of cellular membranes and the cells release blebs possibly without caspase upregulation. DOPC liposomes are hydrolysed by PEI, apparently in a molar mass related manner (Fig. S5), Baciu et al.’s hydrolysis theory appears supported by the available evidence. The issue of toxicity resolves into whether membrane hydrolysis is occurring more rapidly than the cells repair systems, or not. When hydrolysis is more rapid than repair this leads to eventual cell death by lysis of the membrane. If the rate of repair can counterbalance the lysis of membranes the material is tolerated with an observable increase in mitochondrial activity to power the repair.

We have previously suggested that hydrolysis can be used to explain endosomal release without resorting to hydrostatic pressure. (Monnery and Hoogenboom, 2015) In short the polycation-polanyon complex will fall apart when acidified if the pKₐ of the polycation is within the normal range of cells, and the released free polycations will subsequently hydrolyse the phospholipids to induce a pore in the endosome from within. It also raises a potential mechanism for internalization without endocytosis, and release via autophagy.

Table 3
<table>
<thead>
<tr>
<th>Material</th>
<th>MITT 4 h</th>
<th>MITT 24 h</th>
<th>MITT 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (95% CI)</td>
<td>IC₅₀ (95% CI)</td>
<td>IC₅₀ (95% CI)</td>
</tr>
<tr>
<td>PEI34</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PEI65</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PEI136</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PEI222</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PEI358</td>
<td>193.0 (85.7–0.435)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PEI500</td>
<td>66.1 (22.6–193.6)</td>
<td>433.7 (148.2–1270)</td>
<td>&gt;1000</td>
</tr>
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<td>PLL4</td>
<td>&gt;1000</td>
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<td>&gt;1000</td>
</tr>
<tr>
<td>PLL115</td>
<td>9.2 (5.0–17.2)</td>
<td>51.2 (27.5–95.4)</td>
<td>13.6 (7.5–24.5)</td>
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<td>&lt;1</td>
<td>8.3 (4.6–14.9)</td>
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<td>25b-PEI</td>
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<td>141.0 (80.3–247.4)</td>
<td>10.1 (4.7–21.7)</td>
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</tbody>
</table>

Fig. 3. Mitochondrial activity assayed by MTT assay. A549 cells were seeded 16 h before exposure at 10,000 cells per well. Exposure times: (a) 4 h, (b) 24 h, (c) 48 h n = 4.
4. Conclusions

We set out to determine the relationship between the molecular weight of I-PEI (and related polycations) and cytotoxicity, a topic of much speculation but no unambiguous data to date. Assays of cell membrane damage and mitochondrial activity clearly showed a dose-response and importantly a molecular weight response. We have concluded that the cytotoxicity of polycations is directly related to molecular weight and pH, with higher molecular weight and more cationic materials being more toxic. The disruption of cell membranes even by low MW polycations over time indicates there is no such thing as "non-toxic" polycations. Two theories of the mechanism of cellular membranes (hydrolysis and surfactant-effect) have been proposed in the literature, and our observations of cells and liposomes support the hydrolysis theory as first toxicity effect on the cell, as the predicted blebs are observed with cells and DOPC hydrolyses to MOPC and oleic acid in the presence of I-PEI dependent on the molecular weight of the polymer. The mechanism of toxicity appears, thus, to have as first step the hydrolysis of cellular phospholipids. This has implications on polymeric transfection and could provide a potential mechanism of cellular entry without endocytosis.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2017.02.048.

References


Electrochemical paper-based peptide nucleic acid biosensor for detecting human papillomavirus

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**f** Electrochemistry and Optical Spectroscopy Research Unit, Department of Chemistry, Chulalongkorn University, Pathumwan, Bangkok, 10330, Thailand
**g** Nanotec-CU Center of Excellence on Food and Agriculture, Bangkok, 10330, Thailand

**HIGHLIGHTS**
- A paper-based DNA biosensor using AQ-PNA probe and G-PANI modified electrode was first developed.
- This developed DNA biosensor was highly specific over the non-complementary DNA.
- This sensor was successfully applied to detect the HPV-DNA type 16 obtained from cancer cell lines.
- This sensor is inexpensive and disposable, which can be incinerated easily and safely after use.

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Human papillomavirus

**ABSTRACT**

A novel paper-based electrochemical biosensor was developed using an anthraquinone-labeled pyrrolidinyl peptide nucleic acid (acpcPNA) probe (AQ-PNA) and graphene-polyaniline (G-PANI) modified electrode to detect human papillomavirus (HPV). An inkjet printing technique was employed to prepare the paper-based G-PANI-modified working electrode. The AQ-PNA probe baring a negatively charged amino acid at the N-terminus was immobilized onto the electrode surface through electrostatic attraction. Electrochemical impedance spectroscopy (EIS) was used to verify the AQ-PNA immobilization. The paper-based electrochemical DNA biosensor was used to detect a synthetic 14-base oligonucleotide target with a sequence corresponding to human papillomavirus (HPV) type 16 DNA by measuring the electrochemical signal response of the AQ label using square-wave voltammetry before and after hybridization. It was determined that the current signal significantly decreased after the addition of target DNA. This phenomenon is explained by the rigidity of PNA-DNA duplexes, which obstructs the accessibility of electron transfer from the AQ label to the electrode surface. Under optimal conditions, the detection limit of HPV type 16 DNA was found to be 2.3 nM with a linear range of 10–200 nM. The performance of this biosensor on real DNA samples was tested with the detection of PCR-amplified DNA samples from the SiHa cell line. The new method employs an inexpensive and disposable device, which...
1. Introduction

The most important factors for diagnostic devices, especially for developing countries, are low cost, simplicity and speed of results for early screening and monitoring of disease biomarkers. To achieve this goal, paper-based analytical devices (PADs) have been widely used as an alternative device design for point-of-care (POC) applications [1–3]. Two detection modes that have been most frequently used with PADs include colorimetric and electrochemical detections. Since first reported by Dungchai et al. [4], PADs with electrochemical detection (ePADs) have increasingly attracted attention as they offer a combination of simplicity, low power requirements, low limits of detection, and ease of quantitation [5–8]. ePADs are therefore an ideal platform for developing sensitive, selective DNA biosensors for point-of-care applications.

In electrochemical DNA biosensors, many different electrode types have been used, including a gold [9–11], hanging mercury drop (HMDE) [12,13] and various carbon-based materials [14–17]. Carbon is considered a good electrode material due to its low cost, wide potential range, chemical inertness and low background current. Furthermore, carbon electrodes have a fast response time and can be easily fabricated in different configurations. These features make carbon suitable for use in ePAD DNA biosensors. However, the use of micro-scale electrodes as part of ePAD is a major obstacle due to limited sensitivity. To overcome this problem, graphene (G) has been used as a carbon-based nanomaterial and has achieved significant popularity due to the large specific surface area and unique electrochemical properties of G [18–20]. G-based electrodes exhibited superior performance compared to other carbon-based electrodes in terms of their electro-catalytic activity and electrical conductivity [21,22]. G has also been used in combination with various types of functional materials to fabricate high-performance electrodes. Among them, polyaniline (PANI) is a useful conducting polymer that has been widely used for electronic, optical and electrochemical applications such as enzyme-based biosensors and DNA assays due to its excellent environmental stability and unusual doping/dedoping chemistry [23–25]. Moreover, PANI improves the dispersion and reduces the agglomeration of the planar sp²-carbon of G [23,26]. PANI also possesses free amino groups, which can act as a handle for the covalent immobilization of suitable detection probes via amide bonds [27,28]. Finally, doped PANI possesses the positive charge of the amino group, which can immobilize negatively-charged probes via electrostatic interactions. Thus, it is a challenge to evaluate alternative systems for immobilization of various bio-recognition elements via electrostatic interaction.

For most electrochemical DNA biosensors, a probe that is designed to detect a specific sequence of target DNA is first immobilized on the electrode. Then, the electrochemical signal of an electroactive species, which was either covalently attached to the probe or added later as an indicator, is recorded and compared before and after the hybridization with the complementary DNA target [12,29]. The probe is a key parameter that determines the detection selectivity. While most DNA biosensors employ short oligodeoxynucleotide probes, several alternative probes have been used with great success. Peptide nucleic acid (PNA) [30,31], a synthetic DNA mimic with a peptide-like backbone of repeating N-(2-aminoethyl)-glycine units replacing the sugar-phosphate in natural DNA or RNA, has attracted increasing interest as the probe for electrochemical DNA biosensors [12,32–34] due to its sequence-specific binding to DNA or RNA, resistance to nucleases and proteases, and strong binding to the target DNA. Recently, Vilaivan’s group [35–37] proposed a new conformationally constrained pyrrolidinyl PNA system (known as acpcPNA) that possesses an α,β-peptide backbone derived from α-proline/2-aminoacyclopentanecarboxylic acid. This new acpcPNA demonstrates a stronger binding affinity and higher specificity towards complementary DNA compared to DNA and Nielsen’s PNA, and there are several applications of acpcPNA as a probe for DNA biosensors [38–40].

Human papillomavirus or HPV is the common virus that can be passed through any type of sexual contact. There are some high-risk types of HPV including type 16 and 18, which can cause abnormal changes to the cells of the cervix. These changes can lead to cervical cancer which, is one of the most important health problems for women. The mortality occurring from cervical cancer has continuously increased especially in developing countries that have limited medical facilities [41].

In our previous work [38], the electrochemical sensor based-on acpcPNA probe for HPV detection was reported. Although the good limit of detection (LOD) was achieved, the use of PVC-based sensor was costly and the procedure relies on covalent method for probe immobilization was complicated and time-consuming. Cost is the most critical challenge for disposable POC electrochemical sensors. In this work, using PAD has potential to address all of challenges of disposable sensor in term of low-cost and simple fabrication. Electrostatic immobilization is also a key step of this work. This method eliminated the complicate steps of covalent process and significantly reduced time. Moreover, inkjet printing used for electrode modification step is advantageous due to it provided electrode reproducibility and high pattern resolution [42]. Inkjet-printing is scalable of mass production, while small amount of modifier is wasted in the modification process.

Here, we aim to develop a novel paper-based electrochemical DNA biosensor using an AQ-labeled acpcPNA probe in combination with a G-PANI modified electrode. In this new DNA biosensor, the acpcPNA probe labeled with anthraquinone (AQ) was first incorporated onto a G-PANI-modified ePAD using electrostatic immobilization. In the absence of complementary DNA, a strong signal was observed for the AQ. Hybridization with the complementary DNA target resulted in a decrease of the electrochemical signal that linearly correlated with the concentration of the target. The application of the biosensor to the sensitive detection of HPV DNA type 16 is demonstrated. The proposed method is applicable to the screening and monitoring of HPV-DNA type 16 in the primary stage of cervical cancer, a disease that leads to the death of women around the world, particularly in developing countries that have limited resources for public healthcare.

2. Materials and methods

2.1. Chemicals and materials

Graphene (G) was purchased from A.C.S (Medford, USA), easily incinerated after use and is promising for the screening and monitoring of the amount of HPV-DNA type 16 to identify the primary stages of cervical cancer.

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Polyaniline, camphor-10-sulfonic acid (C_{10}H_{16}O_{5}S), glutamic acid and N-methyl-2-pyrrolidone (NMP) were ordered from Sigma Aldrich (St.Louis, USA). Carbon and silver/silver chloride ink were obtained from Gwent group (Torfaen, United Kingdom). The screen-printed block was made by Chaiyaboon Co. Ltd. (Bangkok, Thailand). The labeling electroactive spacers, 4-(anthraquinone-2-oxo)butyric acid, was synthesized as described previously [39]. Analytical grade reagents, including NaCl, KH_{2}PO_{4}, Na_{2}HPO_{4} and KCl, were purchased from Merck and used without further purification. Synthetic oligonucleotides which correspond to partial sequences of HPV type 16 and other types of high risk HPV (HPV types 18, 31 and 33) used in the specificity test, were obtained from Pacific Science (Bangkok, Thailand). The sequences of the DNA oligonucleotides used are shown in Table 1.

The forward (5’-CACCTCCAGC-Lys(AQ)NH_{2}) and reverse primer (5’-GCCTTAAATCCTGCTTGTAG-3’) used for the PCR cell-line samples were purchase from Pacific Science (Bangkok, Thailand). The cervical cancer cell-lines with (SiHa) HPV types 16 were obtained from the Human Genetics Research Group, Department of Botany, Faculty of Science, Chulalongkorn University.

2.2. Apparatus

The electrochemical measurements using square-wave voltammetry (SWV) were performed using a CHI1232A electrochemical analyzer (CH Instruments, USA). A three electrode system was used and the working electrode was a G-PANI-modified screen-printed carbon electrode (4 mm in diameter). The electrochemical impedance spectroscopy (EIS) was carried out using PGSTAT 30 potentiostat (Metrohm Siam Company Ltd., Switzerland) in a solution of 1.0 mM [Fe(CN)_{6}]^{3-/4-} with the frequency range from 0.1 to 10^{-3} Hz. The Dimatix™ Materials Printer (DMP-2800, FUJIFILM Dimatix, Inc., Santa Clara, USA) was used for the electrode modification. JEM-2100 transmission electron microscope (Japan Electron Optics Laboratory Co., Ltd, Japan) was used for characterization of G-PANI composites. MALDI-TOF MS spectra was performed on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany).

2.3. Fabrication of a paper-based electrochemical DNA sensor

A paper-based electrochemical DNA sensor was fabricated using the wax-printing method [43,44]. Initially, the patterned paper was designed using Adobe Illustrator and printed onto the filter paper (Whatman No.1) using a wax printer (Xerox Color Qube 8570, Japan). The printed paper was then placed on a hot plate (175 °C, 50 s) to melt the wax and create a hydrophobic barrier. For electrode fabrication, three electrode systems were prepared using an in-house screen-printing method. First, carbon ink was screened as a working electrode (WE) and counter electrode (CE). Afterward, silver/silver chloride was screened as a reference electrode (RE) and conductive pad. The basic design of the paper-based electrochemical DNA biosensor was shown in Fig. S1.

2.4. Inkjet-printing of a G-PANI composite modified paper-based electrochemical DNA biosensor

Prior to electrode modification, a G-PANI composite was prepared using a physical mixing method following procedures extracted from relevant literature [45]. Graphene powder (10 mg) was dispersed in N-methyl-2-pyrrolidone (NMP) and sonicated for 20 h at room temperature. Subsequently, 20 mg of PANI (emer-didine base) was doped with 25 mg of camphor-10-sulfonic acid (CSA) to generate a positively charged amino group and also dissolved in 10 mL of NMP. For the preparation of the G-PANI conductive ink, the solutions of G and PANI were mixed together and stirred for 1 h. The mixture was centrifuged at 5000 rpm and filtered through a 0.43 µm filter membrane before use. The morphology of G-PANI conductive ink was characterized by transmission electron microscopy (TEM).

For electrode modification, the G-PANI composite solution was used as the ink for inkjet printing. The G-PANI conductive ink was loaded into a cartridge and printed onto the working electrode of the paper-based electrochemical DNA sensor using a Dimatix™ Materials Printer (DMP-2800, FUJIFILM Dimatix, Inc., Santa Clara, USA) at 30 °C, 20 V of applied voltage and 25 µm of drop spacing. Modification of the electrode is shown in Scheme 1A. Six layers of G-PANI composite solution were printed onto the surface area of the working electrode. Next, the modified electrode was heated at 65 °C for 30 min to dry the solvents from the G-PANI conductive ink.

2.5. Synthesis and labeling of the acpcPNA probe

The acpcPNA probe with the sequence Ac-(Glu)_3-CATA-CACCCTCCACGG-lys(AQ)NH_{2} (written in the N→C direction, Ac = acetyl; AQ = anthraquinone; Glu = glutamic acid; Lys = lysine) was designed to detect 14 base synthetic oligonucleotide target with a sequence corresponding to human papillomavirus (HPV) type 16. The probe, referred to as AQ-PNA, was synthesized by solid-phase peptide synthesis using Fmoc chemistry as previously described [35]. The probe was modified with three glutamic acid residues at the N-terminus to incorporate the negative charge, which was followed by end-capping with an acetyl group. At the C-terminus, the acpcPNA probe was labeled with anthraquinone (AQ) via 4-(anthraquinone-2-oxo) butyric acid to the amino side chain of a C-terminal lysine residue. The progress of the reaction was monitored by MALDI-TOF-MS analysis on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The modified PNA on the solid support was treated with 1:1 (v/v) aqueous ammonia:dioxane in a sealed tube at 60 °C overnight to remove the nucleobase protecting groups. Following cleavage from the solid support with trifluoroacetic acid (TFA), the AQ-PNA was purified by reverse-phase HPLC (C18 column, 0.1% (v/v) TFA in H_{2}O-MeOH gradient). The identity of AQ-PNA was verified by MALDI-TOF MS analysis, and the purity was confirmed to be >90% by reverse-phase HPLC.

2.6. Immobilization and hybridization of the PNA probe

First, the AQ-PNA probe (3 µL, 125 nM) was immobilized onto the G-PANI modified screen-printed carbon electrode surface by the drop-casting method and incubated for 15 min at room temperature. Next, 50 µL of PBS was placed onto the electrode surface. After immobilization of the probe, the AQ-PNA/G-PANI/SPCE modified electrode was hybridized with 3 µL of target DNA for 15 min. Then, the electrochemical signal response was measured using square-wave voltammetry. The immobilization and hybridization procedures are illustrated in Scheme 1B.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complementary DNA (HPV type 16)</td>
<td>5’-GCTGCCGCTGTATG-3’</td>
</tr>
<tr>
<td>Non-complementary DNA 1 (HPV type 18)</td>
<td>5’-GGATGCTGCACCGG-3’</td>
</tr>
<tr>
<td>Non-complementary DNA 2 (HPV type 31)</td>
<td>5’-CCAAAACCCCAAGG-3’</td>
</tr>
<tr>
<td>Non-complementary DNA 3 (HPV type 33)</td>
<td>5’-CACATCCACCCGCA-3’</td>
</tr>
</tbody>
</table>
2.7. PCR amplification of the cell line DNA sample

The SiHa (HPV type 16 positive) cell line DNA sample was amplified using PCR as previously reported [38]. Briefly, the amplification mixture containing 0.2 mM deoxynucleotide triphosphate mixture, 1x buffer (KCl, Tris) + 1.5 mM MgCl2, 0.5 U of Taq polymerase, 0.4 µM of each of the primers and 100 ng/µL of the cell line DNA sample was amplified at 95 °C for 10 min, followed by 30 cycles at 52 °C for 30 s and finally 72 °C for 7 min. The success of the PCR was confirmed by gel electrophoresis in 2% (w/v) agarose TBE gel followed by staining with ethidium bromide and visualization under a UV-transilluminator.

2.8. Electrochemical measurement

For electrochemical measurements, the square-wave voltammetry was used throughout the experiment using a potentiostat (CHI1232A). After the immobilization process, 50 µL of PBS was dropped onto the modified working electrode, followed by adding the DNA target. Hybridization with AQ-PNA probe was done for 15 min. The electrochemical detection was performed under the optimal parameters including a frequency of 20 Hz, an amplitude of 100 mV and a step potential of 20 mV. The electrochemical signal after the hybridization was measured and compared with the signal in the absence of the DNA sample.

3. Results and discussion

3.1. Characterization of the G-PANI conductive ink

To confirm the success of the preparation of the G-PANI conductive ink, the morphology of the ink was characterized by transmission electron microscopy (TEM). Fig. S2A shows a TEM image of the G-PANI composite, which indicates a strong dispersion of G inside the composites without aggregation. Moreover, the electron diffraction pattern of G (Fig. S2B) matches very well with the standard, single crystal graphite.

The influence of the G-PANI ratio on the electrochemical conductivity of the modified electrodes was investigated. The anodic current of 1.0 mM [Fe(CN)6]3–/4– increased with an increasing G:PANI ratio. The highest anodic current of [Fe(CN)6]3–/4– was observed with the G-PANI modified electrode at the ratio of 1:2 indicating the optimal sensitivity of the modified sensor (Fig. S3). In addition, the peak currents gradually decrease for the higher G:PANI ratio, which could be due to the agglomeration of G within the G-PANI modifier [45]. Moreover, the effect of the number of printed G-PANI layers was also investigated as shown in Fig. S4. The anodic peak current of 1.0 mM [Fe(CN)6]3–/4– tends to increase with an increase in the number of G-PANI layers. However, the current of the printed G-PANI modified electrode slightly decreased over 6 printing layers. We also believe that this phenomenon arises from the aggregation of excess G on the modified electrode surface causing a decrease in the anodic current response.

Next, the electroactive surface area (A) of SPCE and G-PANI modified electrode were determined to illustrate that the G-PANI conductive ink increases the surface area of electrode, 5.4 times increase of electrode-electroactive surface area, thus, G-PANI conductive ink can improve the surface area of electrode, leading to enhance the sensitivity. As a result, the six G-PANI-layered modified electrode and the 1:2 ratio of G:PANI were considered to be the optimal conditions and were used for subsequent experiments.

3.2. Characterization of labeled acpcPNA probe

In order to confirm the success of the synthesis and labeling of
the AQ-PNA probe, MALDI-TOF mass spectrometry was used to characterize the unlabeled and labeled acpcPNA probes. The 14-mers unlabeled acpcPNA showed a mass peak at 4754 m/z. After labeling, the mass increased to 5477 m/z. This increase of 723 m/z coincides with the mass of AQ and three glutamic acid residues plus an acetyl group. Therefore, the labeling of the acpcPNA probe with the electroactive species and the negatively charged polyglutamate was confirmed.

3.3. Electrochemical characterization

Electrochemical impedance spectroscopy (EIS) was used to characterize the AQ-PNA probe immobilization onto the working electrode. In general, the shape of the semicircle portion obtained from the EIS spectrum relates to either the electron transfer limited process or electron transfer resistance. Fig. 1A shows the Nyquist plots obtained from SPCE, G-PANI/SPCE and AQ-PNA/G-PANI/SPCE before and after hybridization with the target DNA in a solution of 0.1 M [Fe(CN)₆]³⁻/⁴⁻. Fig. 1A (inset) shows the Nyquist curve of unmodified SPCE (red line) with an $R_\text{ct}$ value of 27.5 kΩ. This value is larger than that obtained from G-PANI/SPCE, and indicates that the unmodified SPCE has a higher charge-transfer resistance. Therefore, the decrease in $R_\text{ct}$ to 5.08 kΩ of G-PANI/SPCE (blue line) indicates that G-PANI improves the electron transfer rates. After the immobilization step (Fig. 1A), the semicircular portion of AQ-PNA/G-PANI/SPCE (pink line) dramatically decreased with the $R_\text{ct}$ value of 2.67 kΩ. This result can be explained theoretically as AQ is electroactive and can improve conductivity. Thus, the AQ-labeled PNA probe, which was immobilized onto the electrode surface, can facilitate the electron transfer process at the interface between the G-PANI/SPCE surface and the electrolyte. For this reason, the Nyquist curve of AQ-PNA/G-PANI/SPCE should have possessed the smallest semicircular portion. In order to confirm the success of the immobilization of the AQ-PNA probe, the heterogeneous electron-

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**Fig. 1.** (A) Nyquist plot of SPCE (inset), G-PANI/SPCE (inset), AQ-PNA/G-PANI/SPCE before and after hybridization with target DNA in 0.1 M [Fe(CN)₆]³⁻/⁴⁻. (B) Square-wave voltammograms of immobilized AQ-PNA probe on G-PANI/SPCE before and after hybridization with an equimolar concentration of target DNA.
transfer rate constant ($K_{et}$) value was obtained from Equation (2), which shows the relationship between $R_{ct}$ and $K_{et}$.

$$K_{et} = \frac{RT}{n^2F^2R_{et}AC_{redox}}$$

where $n =$ number of electron transfer, $F =$ faraday’s constant (KJ mol$^{-1}$), $A =$ electrode surface area (cm$^2$), $C_{redox} =$ concentration of the redox couple (mol cm$^{-3}$). From the equation, the $K_{et}$ values for SPCE, G-PANI/SPCE and AQ-PNA/G-PANI/SPCE were calculated to be $7.45 \times 10^{-5}$ cm s$^{-1}$, $40.28 \times 10^{-5}$ cm s$^{-1}$ and $76.70 \times 10^{-5}$ cm s$^{-1}$, respectively. The increasing of $K_{et}$ values inferred that the electron transfer process on AQ-PNA/G-PANI/SPCE is easier and faster than that on G-PANI/SPCE and SPCE. These results indicate that the negatively charged AQ-PNA probe was successfully immobilized onto the positively charged G-PANI modified electrode surface through electrostatic attraction.

Next, the electrochemistry of the immobilized AQ-PNA/G-PANI/SPCE probe before and after hybridization with the DNA target were investigated using square-wave voltammetry (SWV), (Fig. 1B). The immobilization of the AQ-PNA/G-PANI/SPCE probe displayed a redox peak at approximately $-0.65$ V. After hybridization with an equimolar quantity of the complementary target DNA, the electrochemical response significantly decreased due to the increased rigidity of the PNA-DNA duplex relative to the native PNA probe. The rigidity hinders the electron transfer between the redox-active label (AQ) and the electrode surface [46,47].

### 3.4. Optimization of experimental variables

The experimental conditions were optimized next. The effect of
AQ-PNA probe concentration on AQ electrochemical oxidation was investigated first. The oxidation current obtained for different concentrations of the AQ-PNA probe measured by square wave voltammetry are shown in Fig. 2. The current continuously increased up to 125 nM. Above 125 nM, the current decreased significantly. The decrease in peak current at high AQ-PNA concentrations can potentially be caused do to an increased thickness of the organic layer [48,49], which leads to a lower electron transfer between AQ and the working electrode surface. Hence, the probe concentration of 125 nM was selected as the optimal concentration for further experiments.

For electrochemical detection using SWV, the variable parameters including frequency, step potential and amplitude were optimized using a 125 nM AQ-PNA probe concentration without DNA target shown in Fig. S6. The optimal parameters for electrochemical detection of this system was found to be 20 Hz of frequency, 100 mV of amplitude and 20 mV of step potential.

3.5. Analytical performance

To evaluate the analytical performance of the AQ-PNA/G-PANI/SPCE modified electrode, different concentrations of DNA target were determined using SWV analysis. Fig. 3 shows the voltammograms as well as the calibration curve (inset) as a function of target DNA concentrations. The calibration curve provided a linear range from 10 nM to 200 nM with a correlation coefficient of 0.997. The limit of detection (LOD) and limit of quantitation (LOQ), which were calculated as the concentration that produced a signal at 3 times and 10 times of the standard deviation of the blank (N = 5) [4], were found to be 2.3 nM and 7.7 nM, respectively. Our proposed method provides a wide linear range and sufficiently low detection limit for HPV detection. Table S1 shows a comparison of electrochemical performance between AQ-PNA/G-PANI/SPCE and the other modified electrodes used for HPV detection. It can be seen that a sufficiently low detection limit for HPV detection could be obtained from our proposed method. Importantly, this ePAD DNA biosensor can be easily and inexpensively prepared compared to the other HPV DNA biosensors [38,48,50].

3.6. Selectivity of the HPV type 16 detection

In order to investigate the selectivity of the acpCNA probe, the current response obtained from the 14-nucleotide oligomer HPV type 16 DNA target was compared to non-complementary 14-nucleotide oligomers, which originate from the other types of high risk HPV (types 18, 31 and 33), under the same experimental conditions. As shown in Fig. 4, a significantly different current was only obtained from the complementary DNA relative to the non-complementary DNA. Therefore, the immobilized AQ-PNA probe selectively binds to the HPV type 16 DNA target sequences. Accordingly, the proposed DNA biosensor demonstrated high selectivity to HPV type 16 DNA.

3.7. Reproducibility and stability of the paper-based electrochemical DNA biosensor

The electrode-to-electrode reproducibility and stability of the paper-based electrochemical DNA biosensor was examined. The relative standard deviations (RSDs) of five electrodes were tested in the concentration range of 10–200 nM. The % RSDs was determined to be between 2.16% and 7.79%. These results indicate that the proposed DNA biosensor offers acceptable reproducibility. Moreover, the storage stability is another important parameter for DNA biosensor development. The ePAD DNA biosensor was stored at room temperature (25 °C) for 2 weeks before recording the current response to 10 nM target DNA. It was determined that 90.2% of the initial current response was retained in the aged sensor compared to the response obtained from the freshly prepared sensor. The stability of this DNA sensor is mainly attributed to the environmental stability of PANI.

3.8. Detection of the PCR DNA sample

To test the ePAD biosensor, DNA was extracted from the SiHa cell line, HPV type 16 and amplified using PCR. Fig. 5A shows the SWV response of the proposed DNA biosensor in the presence of positive PCR products. It was observed that the current response decreased in the presence of the PCR product from the HPV type 16-positive cell line. Moreover, the current response decreased with increasing amounts of sample, as shown in Fig. 5B. These results indicate that the paper-based electrochemical DNA biosensor has potential for detecting HPV type 16 DNA in PCR samples in clinical samples in future work.

![Fig. 5. Square-wave voltammograms of AQ-PNA/G-PANI/SPCE probe in the presence of PCR-amplified HPV type 16 positive sample from SiHa cell-line.](image-url)
4. Conclusions

A novel paper-based electrochemical DNA biosensor was developed and used for the determination of high-risk HPV type 16 using AQ-PNA probe immobilized on a G-PANI/SPCE modified electrode. The AQ-PNA probe was modified with negatively charged amino acids at the C-terminus to enable electrostatic immobilization on the cationic G-PANI electrode. Using SWV, the electrochemical current decreased after hybridization with the complementary target DNA. Under optimal conditions, a linear range of 10−200 nM was obtained and the limit of detection was determined to be 2.3 nM. The proposed DNA sensor also exhibited very high selectivity against non-complementary 14-base oligonucleotides, including HPV types 18, 31 and 33 DNA. Finally, this sensing system was successfully applied to detect the PCR amplified DNA from HPV type 16 positive SiHa cells. As was demonstrated, several features make this highly sensitive ePAD DNA biosensor suited as an alternative tool for the diagnostic screening and detection of cervical cancer. First, the ePAD can provide a low-cost, disposable sensor for this POC application. Second, the immobilization through electrostatic attraction using G-PANI modified electrode is attractive relative to covalent immobilization because of its inherent simplicity. Third, the inkjet printing method for electrode modification process provides the potential for mass production while helping to reduce the variation among individual sensors, which is crucial for disposable sensor.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2016.11.071.

References


The Fizzling Foam of Champagne

Michèle Vignes-Adler*

"On le mérite en cas de victoire, en cas de défaite on en a besoin", "In victory, you deserve Champagne, in defeat, you need it"
Napoléon Bonaparte

Champagne is definitely associated to Dom Pérignon (1639–1715) who elaborated the “method champenoise”.,[1] By blending three wines (Pinot Noir, Pinot Meunier, and Chardonnay) in a barrel, he obtained a flat “base wine” that he bottled with added sugar and yeast in tightly closed bottles for a second alcoholic fermentation to produce more alcohol and carbon dioxide. This step of the process, called “prise de mousse”, lasts about six weeks and is responsible for the fizzling foam of champagne. The subsequent long ageing on lees (the deposits of dead or residual yeast that sediments out) gives the flavor and bouquet to the champagne. After disgorging expels the lees, the wine is white, limpid (clear), and sparkling, and it is a universal symbol for happiness and success, highly prized for the celebration of very special events.

Actually, the process of making champagne has benefited from technological innovations. The quality of the glass bottles increased almost accidentally when, after the shortage of wood in England owing to the construction of warships, coal replaced wood in the glass furnaces. The result improved the glass annealing and made the bottle’s mechanical strength much greater. Pilgrims of Saint Jalmes’ way brought back stoppers from Galicia, these were made from the bark of cork oaks, and closed the bottles tightly during the second fermentation and ageing on lees. These strong and tightly closed bottles could withstand the high pressure of carbon dioxide generated. Tellier invented the frigorific machine used for the lees disgorging step.

Since then, champagne makers have researched the agronomical, oenological, and biochemical aspects, vines, yeast strains, and malolactic fermentation, to name a few. However, for the demanding consumer, the fizzling foam and the bubbling are definitely the most important organoleptic properties of champagne, even more than the flavor. Generous just after pouring the wine into the flute, the foam should collapse in a few seconds until it remains as a raft of bubbles at the surface of the wine (Figure 1a,b). Then a hole appears at the center of the raft, which quickly expands until it remains solely as a bubble collar at the periphery of the wine surface, fed by trains of bubbles nucleated at some spots of the inner surface of the glass. For full enjoyment, the bubbling should last as long as the conversation about the celebrated event.

In this context, the Moët & Chandon Company decided to investigate the foam and bubbling appearance, and asked physicists to address the following issues:

- How can the champagne foaming properties be investigated systematically?
- Why does champagne from a same bottle bubble nicely in one glass and not in another?
- Why does the foam appearance of champagne differ from those of beer, gaseous water, or soda?
- Of all the chemical compounds in champagne, which ones are foam active?
- What is the origin of the “gushing”, the dramatic formation of foam that may occur as champagne is being bottled on bottle-lines?

To answer these questions, academic scientists have investigated champagne as just another foaming liquid by performing experiments on the surface properties, the films, and bubbles, and the foam. The ultimate purpose was to relate the information gained at different scales. Experiments were performed with champagnes, base wines, and model solutions mimicking the base wines.

The wine of Champagne is an acidic hydro-alcoholic solution that contains a few g L$^{-1}$ of glycerol, tartaric and malic or lactic acids, and amino acids, and at less than g L$^{-1}$ concentrations, mineral ions, organic compounds, and, for example, proteins, glycoproteins, polysaccharides, polyphenols and volatile aromatic substances. The pH value is typically 3.0–3.2 and the ionic strength 0.02 m. It is super-saturated with carbon dioxide, whose partial pressure in the bottleneck is 7 atm at 20°C in thermodynamic equilibrium with its concentration in the wine according to an empirical Henry-like law $P_{CO_2}=K[CO_2]$ where $K$ depends on the temperature, the sugar and ethanol concentrations in the wine.[2]

Upon uncorking and pouring the champagne into the flute, the CO$_2$ pressure in the wine abruptly decreases to 1 atm, and to restore the thermodynamic equilibrium, the champagne degases. The generous initial foam is due mostly to the CO$_2$ molecules in the bottleneck. Degassing of the wine...

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occurs by diffusion of dissolved CO₂ molecules across the wine surface, while 20% is due to bubbling. Bubbling results from the nucleation of bubbles which have a radius larger than a critical value, which depends on the liquid surface tension and the gas supersaturation. Actually, champagne is only weakly supersaturated with CO₂ and neither homogeneous nor heterogeneous bubble nucleation occurs spontaneously; this would necessitate unrealistic supersaturation (1000 atm) to overcome the energy barrier. A simple thermodynamic calculation shows that bubbles can only nucleate within the liquid at long and narrow pre-existing metastable gas cavities, with a radius of curvature larger than the nucleation value of 0.15 \( \mu m \) in champagne. In 1996, Robillard and Lehuèdé discovered that bubbling sites were either fibers or calcareous protuberances (Figure 1c). By chemical acidic cleaning in a dust-free laboratory white room, they very easily suppressed the bubbling. This explains why no bubbling is observed with some flutes, which presumably have molecularly smooth inner surface.

Two processes destabilize foams: 1) ripening, which occurs as smaller bubbles empty into larger neighboring ones, because of a larger capillary pressure (Laplace’s law), 2) film rupturing, caused by a local excessive thinning and an insufficient quantity of adsorbed compounds to prevent it. Ripening can be hindered by addition of a very small amount of insoluble gas. Silicone particles from lipstick and fatty particles from cream cakes are foam killers: they spread on the film surfaces and drag some adjacent film liquid with them, the film thins locally so that unbalanced attractive van der Waals intermolecular forces cause its rupture. Conversely, foam stability can be positively modified by surface-active macromolecules. Using a sparging method, Malvy et al. measured foamability and foaminess, of mixtures of champagne and various proportions of its ultrafiltrate and ultra-concentrate, obtained by passing it through a molecular sieve (MWCO = 10 kDa). The more ultrafiltered wine in the mixture, the less surface-active compounds, and the lower the foaminess is and vice-versa. Moreover, Senée et al. also showed that the foam parameters of wines are vintage dependent.

Some of the champagne compounds may show surface activity by themselves, for example, proteins, glycoproteins, and esters, or by association with other compounds (polysaccharides associated with proteins, for example). However, they are only present in small quantities, as demonstrated on a local scale by Liger-Belair et al. who measured the velocity of a bubble growing from the dissolved CO₂ molecules rising in glasses of champagne and in beer. In champagne, the velocity follows a Hadamard–Rybczinski law, whereas it follows a Stokes law in beer. Unlike the beer bubble, which behaves like a solid sphere, which indicates there are many adsorbed materials rigidifying the surface of the beer bubbles and enhancing the foam stability.

To analyze the action of the surface-active compounds, Senée et al. investigated by optical micro-interferometry the drainage of the film formed above a bubble attached to the surface of base wines in a container (Figure 2). When the film thickness is about 100 nm, small aggregates appear progressively as small black spots in the film. The wine films continue to drain slowly but the mobility and growth aspects
of the aggregates are drastically vintage dependent. With the 1990 base wine (Figure 2), the aggregates become organized in regular patterns, which gives a grainy aspect to the films. With the 1994 base wine, the aggregates move in the film forming dendritic patterns typical of the diffusion growth of bidimensional aggregates. In the closed container, the films do not rupture whereas similar films formed from simple hydro-alcoholic solutions rupture in less than 6 s. They rupture in 25 s and in 50 s for the 1990 and 1994 wines, respectively, if the container is open. The final film equilibrium thicknesses also change with the vintages, 16 nm for the 1990 wine and 35 nm for the 1994 one. Differences in the film equilibrium thickness are assigned to the different behavior of the aggregates in the film. It should be emphasized that no aggregates are present in the bulk liquids. Senéé et al. repeated the experiments with model hydro-alcoholic solutions of yeast glycoproteins (YGP), which are the dominant macromolecules in the foam of champagne[11] with same alcohol content, pH value, and ionic strength as a typical base wine.[9] Remarkably, films from the model alcoholic solution of YGP at 3 mgL\(^{-1}\) concentration, drain like the 1990 wine, with aggregates organized in regular patterns. Their global foam parameters are also equal.

Now, surface tensions of base wines are only 2 mNm\(^{-1}\) lower than that of a hydro-alcoholic solution with the same alcohol content (ca. 48.5 mNm\(^{-1}\)), which means that the wine values are due mainly to the alcohol. Interpretation of surface tensions of YGP solutions with and without alcohol strongly suggests that there is a competition between the ethanol molecules and YGP macromolecules in the adsorption process, with the alcohol hindering the YGP adsorption.

The origin of the aggregates could then be identified. It is unlikely that they are made of proteins. Proteins are highly denatured by ethanol, and particularly so at the protein isoelectric point when the ionic forces are weak. Ethanol acts primarily by weakening the hydrophobic bonds, and exposing the hydrophobic side chains to ethanol in the denatured state.[12] The protein adsorbability is significantly modified in the presence of ethanol. It is likely that proteins are precipitated in the bottle and are then expelled with the lees. The situation is very different for glycoproteins. YGP are essentially mixtures of mannoproteins and glucanes, the proportion of sugar is higher than 90% and the molecular weight of each structure ranges from 40 kDa to more than 100 kDa. They are produced during the alcoholic fermentation and also by the autolysis of dead yeast cells as the champagne is aged on the lees.[13] The hydrophilic carbohydrates tend to protect proteinic moieties against denaturation by ethanol and to increase their solubility; the degree of the protection is, to a first approximation, a function of the number of hydroxy groups. Hence, it can be expected that the interactions between glycoproteins and the alcoholic solvent are intricately dependent on the quality and the sugar content of the glycoproteins, which changes with the vintage.

The aggregates are visible when the film thickness decreases to a value ranging between 50 nm and 100 nm. This value is comparable or smaller than the hydrodynamic diameter 120 nm of the YGP in the solution. A film is a confined system, which hinders Brownian motion in its reduced dimension, and which squeezes the polymeric sugar chains during its thinning. Solvent depletion and increase in the YGP volume concentration result. These processes occur as if the macromolecular concentration increases to a limiting value, corresponding to a supersaturation level where spontaneous precipitation occurs and flat-shaped microgels, with a diameter of approximately 6 \(\mu\)m and a thickness of approximately 15 nm, are formed. The presence of these microgels clearly prevents or slows down excessive thinning of the films, even when the container was open, which

![Figure 2. Top views of films formed from two base wines and a model yeast glycoprotein solution on top of a bubble attached to a wine surface (Experiments were performed in early 1996).](image-url)
stabilizes the lifetime of the films and consequently of the bubble.

On bottling lines for magnum bottles, an excessive volume of foam larger than the 6–8 cm$^3$ volume of the bottleneck occasionally springs up from the bottom of the bottle. Just as for the bubbling in the flute, a pre-existing gas in a long and narrow cavity with low surface energy is necessary for bubble formation at the typical CO$_2$ saturation prevailing in champagnes. A few bubbles are unable to generate such a violent effect. Bottles are fabricated throughout whole year whereas they are only filled during a short period of the year. During storage, the bottle glass evolves and its inner surface attains a whitish color arising from the formation of crystals of sodium and calcium carbonates. Crystals of sodium carbonate are soluble in water whereas calcium carbonate crystals are only soluble in acidic solutions, such as champagne. Valant$^{[14]}$ observed that large crystals of calcium carbonate are cracked (Figure 1d). The cracks can trap air bubbles with a 0.1 $\mu$m radius of curvature, which is smaller than the local critical radius of nucleation (0.5 $\mu$m). However, the dissolution of sodium carbonate and calcium carbonate close to the crack locally increases the CO$_2$ concentration, which locally increases the supersaturation and decreases the nucleation radius. The origin of gushing is thus to be found in chemically aided, non-classical heteronucleation.

The identification of the foam-active compounds in wine was a difficult task, mainly because wines are natural products whose resulting fine flavor and foam aspect depend upon many uncontrollable external factors, such as sunshine and precipitation during vine blossoming and ripening. Oenologists tend to hide these subtleties behind the “vintage” concept, and they only make taste assessments to appreciate wine quality. All base wines of champagne have essentially the same values of alcohol concentration, pH value, and ionic strength. The differences among the various vintages are related mostly to compounds existing in microscopic amounts, for example, organic volatile compounds, proteins and glycoproteins, and polyphenols. Their quality and concentration can vary significantly with the vintages. Laboratory experiments have already greatly enhanced the understanding of these differences by pointing out the role of the yeast glycoproteins, and explain why champagne foams can significantly change with the vintage.

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Characterization of Salophen Co(III) Acetate Ionophore for Nitrite Recognition

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The ionophore [salph]Co(III)OAc is used here for the first time as a recognition molecule for nitrite in lipophilic membranes. The nature of the recognition process is evaluated by UV-Vis, ATR-IR and potentiometry. While the purity of the compound is confirmed by LC-MS and cyclic voltammetry. Experimental evidence suggests a replacement of the acetate ligand by nitrite. The formation constant between [salph]Co(III)OAc and NO2−, β = 10^4, is estimated potentiometrically with the sandwich membrane method. The analytical parameters, LOD = 3 μM, linear range of four orders of magnitude, stability over four days, and the selectivity over chloride (log K_{NO2,Cl} = 10.4) are similar to previous ionophores for the same ion. Undiluted urine samples were spiked with incremental concentrations of nitrite to characterize the matrix effect. Beyond the observed analytical performance, we aim here to offer a rational study to understand an unconventional charged ionophore recognition process.

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1. INTRODUCTION

Anion determination by ionophore-based membrane remains an important challenge in the electrochemical sciences. While a great number of contributions have been made in the past decades, it has so far remained difficult to deviate from the Hofmeister selectivity sequence and to discriminate highly hydrophilic ions from lipophilic ones [1–4]. The difficulty in obtaining ionophores with similar properties as the ones developed for cations results from their intrinsic nature. Anions are larger molecules than cations, and the lower charge to radius ratio results in a less effective electrostatic binding. Different geometric spaces such as spherical, linear, trigonal planar, tetrahedral and octahedral may be found, which require a higher degree of design to make receptors complementary to their anionic guest. The significant hydrophobicity of some anions (i.e., HPO_{4}^{2−}, AcO−, NO_{2}−, NO_{3}−, Cl− and F−) effectively counteracts the host-guest interaction so that their extraction selectivity remains often poor in comparison with hydrophobic anions such as ClO_{4}− or SCN−, which may shed their hydration layer much more easily. Hydroxide ions may often compete quite effectively with anion receptors, resulting in serious interference in all but rather acidic solution conditions. These disadvantages often translate in a less attractive selectivity compared to what is achievable with cation-selective membranes [5].

Among the scientific articles published so far, it is worth mentioning Al(III) and Zr(IV) complexes for fluoride recognition, Co(III) and Co(II) complexes for iodide detection and more specifically Rh(III) salen, salophen, Co(II) salen [6,7], lipophilic Co(II) salophen [8], Co(II) porphyrine [29] and UO_{2} salophen complexes for nitrite detection. In addition another structures with cobalt as a metallic center have been explored such as Co(III) phthalocyanines [10], Co(III) cyanobenzates and Co(III) corroles [3]. Fig. 1 shows the representative ionophore structures for nitrite reported so far. Slight differences are observed in the key analytical characteristics of limit of detection (1−20 μM) and dynamic linear range (4 ± 1 units). To our surprise, formation constant values have typically not been reported for the listed ionophores.

As an illustration of the mentioned issue above, we prepared a new lipophilic ionophore, which in principle is suitable for nitrite determination. It is well established that nitrite is critically important for environmental analysis, food quality control and clinical applications [11,12]. The proposed ionophore belongs to the salophene family where two nitrogens are linked with an aromatic cycle. Such ionophores exhibit the same tetradequate Schiff base...
Fig. 1. Reported nitrite ionophores. I: (-5.1, -5 to -1) [9]; II: (-6.0, -6 to -1) [7]; III: (-6.0, -5 to -1) [6]; IV: (-6.0, -5 to -1) [8]; V: (-6.0, -5 to -1) [38]; VI: (-6.5, -5 to -1) [39]; VII: (-6.0, -5 to -1) [40]; VIII: (-6.5, -5 to -1) [10]. The LOD and linear logarithmic concentration range are reported between parentheses.

properties, allowing them to bind to a wide range of metals, which enables ion-recognition and enantioselective catalytic properties [13]. More specifically, this ionophore is a square planar cobalt salophen with an axial acetate ligand. As a result of this acetate group, the cobalt oxidation state becomes (3+). The characterization offered here aims to elucidate the interaction mechanism between ionophore and nitrite in PVC–DOS membranes. Among the techniques used here, ATR gives us information about the “in-situ” processes that occur in the membrane phase. In other reports, ATR-IR was primarily used for monitoring water penetration into the liquids membranes, but rarely for monitoring reaction processes within membranes [14–19]. Other techniques such UV-Vis cyclic voltammetry and potentiometry were used for validating the proper functioning of the proposed sensor. Beyond the performance of the potentiometric sensor, our key aim was the characterization of the ionophore.

2. EXPERIMENTAL SECTION

2.1. Materials, chemicals and solutions

High molecular weight poly (vinyl chloride) PVC, tridodecylmethylammonium chloride (TDMAC), bis(2-ethylhexyl) sebacate (DOS), N,N’-bis-(3,5-di-tert-butylsalicylidene)-1,2-phenylenediaminocobalt, methylene chloride, tridodecylmethylammonium chloride (TDMAC), Potassium tetrakis[3,5-bis (trifluoromethyl) phenyl]borate (KTFPB), hydrobromic acid (HBr), dichloromethane, tetrahydrofuran (THF), sodium chloride, sodium nitrite, sodium sulphate, sodium acetate, sodium perchlorate, sodium bromide, tetrabutylammonium hexafluorophosphate (TBAH) and tetradeckylammonium tetrakis (4-chlorophenyl) borate (ETH 500) were purchased from Sigma Aldrich (Analytical purity). The monomers methyl methacrylate, 99.5%, (MMA) and n-decyl methacrylate, 99%, (DMA) were obtained from Polysciences Inc. (Warrington, PA). The polymerization initiator 2,2-azobis (isobutyronitrile) (AIBN), 98%, was obtained from Aldrich. Ethyl acetate, dichloromethane 1,4-dioxane, benzene, and anhydrous Na2SO4 were reagent grade from Fisher. Aqueous solutions were prepared by dissolution of salts dissolved in Milli-Q water (>18.2 MΩ cm).

2.2. Electrochemical Equipment

A double-junction Ag/AgCl/3 M KCl/1 M LiOAc reference electrode was used in potentiometric measurements (Model 6.0726.100 Mettler Toledo AG, Schwerzenbach, Switzerland). A platinum-working rod (3.2 cm² surface area) and a glassy carbon rod of (3.0 ± 0.1) mm electrode diameter (7.06 mm² surface area) were used as counter electrode and working electrode respectively, in cyclic voltammetry. Electrode bodies (Oesch Sensor Technology, Sargans, Switzerland) were used to mount the polymeric
membranes. Selectivity coefficients and calibration curves were conducted by zero current potentiometry employing a high impedance input 16-channel EMF monitor (Lawson Laboratories, Inc. Malvern, PA). Measurements of pH were performed with a Metrohm 744 pH meter (Metrohm Autolab, Utrecht, The Netherlands). The cyclic voltammetric measurements were performed with an μ-Autolab System (Metrohm Autolab, Utrecht, The Netherlands) controlled by a personal computer using Nova 1.8 software.

2.3. Ionophore synthesis

In order to prepare (N,N'-bis-(3,5-di-tert-butylsalicylidene)-1,2-phenylenediaminocobalt acetate (Fig. 2, compound a), (N,N'-bis-(3,5-di-tert-butylsalicylidene)-1,2-phenylenediaminocobalt (100 g, 1.67 mmol) dissolved in methylene chloride (10 mL) and acetic acid (0.100 mL, 1.75 mmol). The resulting solution was stirred open to air while solvent was allowed to evaporate, yielding a bright red powder. The powder was washed with hexane and dried (1.1 g, 97% yield) [13].

2.4. Membrane Preparation

Nitrite PVC-based membranes were prepared in the classical manner using an appropriate mass ratio of PVC and plasticizer (%wt. ratio 1:2). Determined amount of nitrite ionophore, TDMAC or NaTFPB, PVC, and plasticizer were dissolved in THF (see Table 1 for the compositions). Afterwards, each cocktail was poured into a glass ring (24 mm ID) affixed onto a glass slide. The organic solution was allowed to evaporate overnight. The thicknesses of the resulting membranes were ca. 0.20 mm. This initial membrane was cut with a hole-puncher into small disks (8 mm diameter) and mounted into the Ostec ion-selective electrode body. Membranes were conditioned in 10⁻³ M sodium nitrite solution for 24 h before measuring them.

2.5. Electrochemical measurements

All electromotive forces (EMF) were recorded at room temperature (25°C) in stirred solutions. A double-junction reference electrode (RE) was employed. Each measurement was performed simultaneously on three electrodes from the same original membrane. Activity coefficients were calculated using the Debye–Hückel approximation. Selectivity coefficients were determined by the separate solutions method (SSM). Cyclic voltammetric studies of 10⁻⁴ M of (N,N'-bis-(3,5-di-tert-butylsalicylidene)-1,2-phenylenediaminocobalt acetate in DMF were performed at the surface of GC versus Ag/AgCl as a reference electrode. The scanning electrode potential was between −1.5 and +0.5 V with a scan rate of 100 mV s⁻¹.

2.6. ATR details

ATR-IR spectra were recorded using a Bruker VERTEX 80v Fourier transform infrared (FT-IR) spectrometer equipped with a liquid nitrogen-cooled narrow-band mercury cadmium telluride (MCT) detector. Spectra were recorded at a resolution of 4 cm⁻¹. For ATR experiments a dedicated flow-through cell was used made from a Teflon piece and a fused silica plate (64 mm-30 mm-5 mm) with holes for the inlet and the outlet (39 mm apart), and a Viton seal (1 mm). The volume of the used flow-through cell is 0.129 mL with a gap of 270 mm. The cell was mounted on an attachment for ATR measurements within the sample compartment of the FTIR spectrometer. The solutions were passed through the cell and over the ZnSe crystal at a flow rate of 0.1 mL min⁻¹ by means of a peristaltic pump (Ismatec, Reglo 100) located in front of the cell. All experiments were performed at room temperature and the spectrometer was evacuated to avoid contributions from gas-phase water and CO₂ [20].

2.7. UV-measurements

The absorbance was measured with a UV–Vis spectrometer (SPECORD 250 plus, Analytic Jena, AG, Germany) in a quartz cuvette (1 cm path length).

2.8. Mass spectra

The mass spectra were acquired by LC/MS systems, API 150EX (AB Applied Biosystems MDS SCIEA, Switzerland). The mass spectrum of (salp)Co(III)OAc in both negative and positive modes in CH₂Cl₂ is shown in Fig. 1S. Unfortunately, either (salp)Co(III) OAc peak or its fragments does not appear in that conditions. Increasing the ionization of (salp)Co(III)OAc was achieved by

Fig. 2. a) (salp)Co(III)X structure (X = AcO, OAc c and d) possible mechanisms of NO₂⁻ recognition (X = AcO⁻, Y = NO₂⁻).
adding a small amount of HBr (1%). The ideal m/z peak for (salph)Co(III)OAc must be 656.75 g mol⁻¹. However, the main observed peak appear at m/z 597.7 g mol⁻¹, which is explained as a sub-fragment [(salph)Co(III)]⁺ of the [(salph)Co(III)]OAc after losing the AcO⁻ group (59.05 g mol⁻¹).

3. RESULTS AND DISCUSSION

The aim of this work is the elucidation of the recognition mechanism of a novel ionophore selective for nitrite. The synthesis of a square planar cobalt salphen with an axial acetate (salph)Co(III)OAc (Fig. 2a) was performed by oxidation of its precursor (salph)Co(II) (Fig. 2a) with acetic acid and acetic anhydride (see experimental section). As suggested in the literature, (salph)Co(III) compounds exhibit a higher chemical stability than (salph)Co(II) derivatives over time.

In order to fabricate a nitrite selective electrode, a specified amount of (salph)Co(III)OAc was introduced in polymeric PVC membranes of established composition (DOS/PVC, ratio 2:1). The knowledge of the effective net charge of the ionophore is important, as it has significant connotations on the membrane sensing mechanism. Ion-selective membranes work on the basis of ion partition between an organic (membrane) and and aqueous phase. For neutral ionophores, the addition of an ion exchanger is a requirement to endow the membrane with permeselectivity (Donnan exclusion properties). Perceptibly, this is not the case for the investigated ionophore (see discussion on potentiometric characteristics below) [21–23].

Fig. 2 illustrates three possible recognition routes between the ionophore and the nitrite species in a lipophilic environment such as bis(2-ethylhexyl) sebacate (DOS, log P = 10.1). The compound b originates as a result of a nucleophilic substitution of NO₂⁻ (labeled as Y) to the opposite axial position followed by AcO⁻ (labeled as X) release. In principle, this mechanism should be interpreted as an anion exchange process where AcO⁻ is replaced by NO₂⁻ ([(salph)CoOAc] to (salph)Co(NO₂). As a result, the ionophore has an intrinsic double function as ion-exchanger and ion receptor.

Similarly, the compounds c and d form by NO₂⁻ substitution. In contrast to b, the resulting c and d adducts exhibit a net negative charge. The main difference between c and d corresponds to a second NO₂⁻ ligand in place of the released AcO⁻ (d). Obviously, c and d suggest an ionophore mechanism analogous to electrically neutral ionophores (i.e., valinomycin for potassium detection). Accordingly, a lipophilic anion exchanger (i.e., TDMAC, TDA, R⁺) must be introduced in the membrane to obtain a selective potentiometric response towards NO₂⁻. For this reason, different experiments (ATR, UV, cyclic voltammetry, potentiometry) were performed to elucidate the proper mechanism.

The (salph)Co(III)OAc interaction with NO₂⁻ was first confirmed by UV–vis cuvette experiments (see Fig. 3). A specified concentration of ionophore (5 × 10⁻⁴ M) was dissolved in THF and the respective absorption spectrum was acquired. Two main bands were observed for the unreacted ionophore at 400 nm and 500 nm. As suggested elsewhere [7] for similar salphen structures, these two bands correspond to the electronic transition n→π*.

In another experiment the same concentration of ionophore was mixed with NO₂⁻ salt (5 × 10⁻⁴ M and 1:1 molar ratio), resulting in a blue shift of 50 nm for both peaks. This suggests a change of the electronic configuration of the ionophore provoked by NO₂⁻ substitution. Similarly, chloride (5 × 10⁻⁴ M) and nitrate anions (5 × 10⁻⁴ M) were also evaluated. The spectrum did not exhibit appreciable changes, indicating a lack of interaction. Control experiments with NO₂⁻ (5 × 10⁻⁴ M) and AcO⁻ (5 × 10⁻⁴ M) did not show any significant absorption in the spectral window. In summary, UV–vis experiments strongly indicate that (salph)Co(III)OAc has a remarkable affinity for NO₂⁻.

The ionophore mechanism was further elucidated by time dependent infrared spectroscopy for an ionophore based PVC membrane, using attenuated total reflection (ATR). This allowed us to measure thin layer membranes while preserving adequate sensitivity and an attractive signal/noise ratio. The penetration depth into the membrane depends exclusively on the selected crystal. In our case the penetration depth corresponds to a few μm (0.5–2.0) given by the ZnSe ATR crystal. To simplify the experiment, a thicker membrane (10 μm) than the penetration depth was chosen. Using this particular condition, the beam is always confined in the membrane phase and contributions from the aqueous phase may be neglected.

Two membrane cocktails were prepared (with and without ionophore) and subsequently deposited onto the same crystal. Afterwards, water was introduced to the ATR cell by a peristaltic pump at a constant flow rate of (0.1 mL min⁻¹) for 10 min and one

---

**Table 1**

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Ionophore (mM)</th>
<th>KTFBP (mM)</th>
<th>TDMAC (mM)</th>
<th>Slope (mV dec⁻¹)</th>
<th>LOD (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>58.5 ± 0.9</td>
<td>-5.3</td>
</tr>
<tr>
<td>M2</td>
<td>15</td>
<td>1.5</td>
<td>0</td>
<td>58.3 ± 1.1</td>
<td>-5.4</td>
</tr>
<tr>
<td>M3</td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>57.2 ± 6.8</td>
<td>-5.5</td>
</tr>
<tr>
<td>M4</td>
<td>15</td>
<td>7.5</td>
<td>0</td>
<td>54.2 ± 2.1</td>
<td>-4.6</td>
</tr>
<tr>
<td>M5</td>
<td>15</td>
<td>1.5</td>
<td>1.5</td>
<td>57.9 ± 0.9</td>
<td>-5.0</td>
</tr>
<tr>
<td>M6</td>
<td>15</td>
<td>0</td>
<td>3</td>
<td>56.8 ± 1.4</td>
<td>-4.8</td>
</tr>
<tr>
<td>M7</td>
<td>15</td>
<td>0</td>
<td>7.5</td>
<td>58.8 ± 0.5</td>
<td>-4.6</td>
</tr>
<tr>
<td>M8</td>
<td>0</td>
<td>0</td>
<td>7.5</td>
<td>58.8 ± 0.5</td>
<td>-4.0</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** UV–vis spectra for: 5 × 10⁻⁴ M (salph)Co(III)OAc (two peaks at 400 and 500 nm) (dash line). b) 5 × 10⁻⁴ M (salph)Co(III)OAc + 5 × 10⁻⁴ M NaNO₂ (two peaks at 370 and 450 nm) (dotted line). c) 5 × 10⁻⁴ M (salph)Co(III)OAc + 5 × 10⁻⁴ M NaCl (two peaks at 400 and 500 nm) (solid line). d) 5 × 10⁻⁴ M NaNO₂. All solutions were prepared in THF mimicking the membrane phase.
intensity at 1539 cm\(^{-1}\) gradually decreases until disappearance (Fig. 4a). Simultaneously, the \(\text{NO}_2^-\) band intensity at 1215 cm\(^{-1}\) starts to appear after the first ten minutes of pumping and subsequently increases up to a stable plateau. The normalized absorbance difference is plotted as function of time, see Fig. 4c, and suggests an exchange of \(\text{AcO}^-\) by \(\text{NO}_2^-\) in the membrane phase mediated by the ionophore. Similar experiments were performed with 1 mM NaCl instead of \(\text{NaNO}_2\) with a fresh membrane. However, in this case, the \(\text{AcO}^-\) band remained unaltered (data not shown). In another control experiment (no ionophore in the membrane), the spectra remain practically constant in the interested wavelength region (data not shown), suggesting that \(\text{NaNO}_2\) co-extraction into the membrane phase can be excluded. According to the ATR results, the mechanism seems to be either b or d, excluding the c alternative.

With the purpose of obtaining more evidence about the ionophore structure and purity, the system was studied further by cyclic voltammetry. The ionophore contains a discrete cobalt moiety, which can be oxidized or reduced by controlled potential techniques in organic solvents. Fig. 5 shows cyclic voltammograms of a range of concentrations of ionophore in DMF using a 0.1 M background electrolyte (tetrabutylammonium hexafluorophosphate, TBAH) after three scans \([8,11]\). The backward and forward scans were performed from 0.5 to \(-1.5\) V (anodic sweep) and from \(-1.5\) V to 0.5 V (cathodic sweep). In the backward scan, the reduction process is attributed to two redox potentials, including 0.2 V for \(\text{Co}^{3+}/\text{Co}^{2+}\) and \(-1.2\) V for \(\text{Co}^{2+}/\text{Co}^{+}\) (Fig. 4). In the forward scan, the peak at \(-1.1\) V corresponds to the reversible couple of \(\text{Co}^{2+}/\text{Co}^{3+}\). On the other hand, the single peak at 0.25 V is attributed to \(\text{Co}^{2+}/\text{Co}^{3+}\) oxidation. This result is in agreement with similar structures already reported \([8,11]\). Additionally, no appreciable oxidation related to \(\text{Co}^{3+}/\text{Co}^{4+}\) is observed for the initial scan confirming the presence of \(\text{Co}^{2+}\) in our initial compound (Fig. 4S). Note that the reduction of \(\text{Co}^{3+}\) to \(\text{Co}^{2+}\) is not completely reversible at 0.2 V. As a consequence, a new peak is observed at \(-0.7\) V that is presumably attributed to a follow-up step of the product originating at 0.2 V.

The determination of complex formation constants in the membrane phase allows us to understand the magnitude of the binding strength between (salphb)Co\(^{3+}\)OAc and \(\text{NO}_2^-\). The so-called sandwich membrane method is very useful in the study of such formation constants \([21,24,25]\). Two membranes, one with
ionophore and ion-exchanger and another one containing only ion-exchanger without ionophore, were prepared. Both membranes were conditioned separately in NO$_2^-$ for 12 h. One of the membranes was then placed on top of the other (labeled as fused membranes) and the potential across the sandwich was measured potentiometrically [26]. Fig. 6 shows the drop of potential (350 mV) that gives information about the ionophore diffusion and complex formation constant (log $B_{L_{i}NO_{2}^{-}} = 7.92$, see supporting information for the calculation) [5].

The influence of pH on the potentiometric responses were tested in 1 mM nitrite solutions for membrane M1 (Fig. 7). A plateau between pH 4 and 6 was observed, suggesting no influence of pH on the electrode response in this region. The potential of electrode decreases at pH higher than 6 owing to OH$^-$ interference [6,8]. The EMF response increased at pH lower than 4, reflecting the speciation of the nitrate ion. A suitable working pH range for the sensor is between 4 to 6 because in this range the electrode shows Nernstian potentiometric response, which is in agreement with the literature [6].

Nitrite calibration curves were performed for membrane M1 in the range of $10^{-7}$ to $10^{-2}$ M. Fig. 8a shows the time traces as a function of successive additions of 1 M NaNO$_2$ stock solution to the initial volume of 100 mL. The resulting emf values are plotted as a function of logarithmic nitrite activity in Fig. 8b. Because the response time is longer than for other membranes (on the order of few seconds) [27], an average of ten points in the interval of 110–120 s (steady state) was used for the calibration curve. A nernstian slope was obtained in the range of $10^{-5}$ to $10^{-3}$ M (58.5±0.9 mV dec$^{-1}$) with a LOD of $10^{-5.3}$. At first glance, this potentiometric data suggests an appropriate sensing ability of the membrane in absence of lipophilic ionic sites. This is agreement with mechanism (1b).

As is well known, bare PVC powder contains a certain amount of anionic sites (around 0.1 mM) [28] that may give a biased interpretation of the ionophore mechanism. Taking into account this effect, membranes without lipophilic ionic impurities such as acrylic (MMA-DMA) [29–35] and polypropylene (PP) [12,36] were prepared to obtain an improved basis for the evaluation of the ionophore mechanism. Fig. 5S and 6S show both MMA-DMA and PP membrane responses, respectively. Nernstian responses are observed for both cases. The detection limits for PP and MMA-DMA membrane (−5.0 and −5.5) were very similar compared to PVC membrane (−5.3). It is worth mentioning that the response of PP membranes were not very stable over time for this particular ionophore (lifetime of 1 d), but the response time was significantly faster. In contrast, MMA-DMA membranes were more stable than PP (at least a week), however, the noise levels increased considerably even after adding ETH500 (Fig. 5S). A compromise

![Fig. 6. Sandwich membrane experiment. Fused membrane is composed of membrane without ionophore (membrane with ionophore. Sample and inner electrolyte are 10 mM NaNO$_2$ + 0.1 mM NaCl.](image)

![Fig. 7. Optimization of pH working range for M1. pH was adjusted by adding diluted sodium hydroxide or hydrochloric acid solutions. The inner and sample solution was composed of 1 mM NaNO$_2$ and 0.1 mM NaCl.](image)

![Fig. 8. Potentiometric time trace for M1. The inner and sample solution was composed of 1 mM NaNO$_2$ and 0.1 mM NaCl. Inset: Calibration curve as a function of logarithmic nitrite activity.](image)
situation, regarding the simplicity of the membrane preparation, stability, LOD and response time, is still found with PVC membranes. With the observed nernstian response slopes without addition of lipophilic sites, a charge ionophore mechanism is, in principle, suggested.

A systematic experimental design was explored in order to identify the influence of anionic and cationic lipophilic sites on the potentiometric response. Charged ionophores exhibit both ionophore and ion-exchanger properties. However, if an ion-selective electrode is fabricated with membranes that only contain the charged ionophore, the observed selectivity is generally not optimal. Indeed, the presence of a cation exchanger may improve the Nernstian response and selectivity because of the stabilization of charged ionophore in the membrane (for more details about this mechanism, see reference [22] and [23]). The ion selective membrane was based on a PVC matrix and the composition of membrane was 66 wt% DOS, 33 wt% PVC, 1 wt% ionophore and ionic lipophilic salt [22]. Table 1 summarizes the membrane compositions. Basically, a gradual concentration increase of either TFPB$^-$ (M2, M3 and M4) or TDMA$^+$ (M5, M6 and M7) was examined.

On the one hand, large amounts of TFPB$^-$ (M4) result in a reduction of the nernstian response towards NO$_2^-$ (about 56 mV dec$^{-1}$ with 10$^{-6}$ M LOD for M4). Evidently, the membrane started to lose perm-selective properties at this concentration. At lower TFPB$^-$ levels (M2 and M3) the membrane exhibited an adequate nernstian response slope. In addition, there is no significant difference between the potentiometric response of M1 and M2 or M3. On the other hand, by adding incremental amounts of TDMAC, the nernstian response was not significantly altered with respect to M1.

Selectivity coefficients were calculated according to the modified separate solutions method, and the obtained values are shown in Fig. 9 and Table 1S. Membranes were always conditioned in the more discriminated anion such as 10$^{-3}$ M sodium sulfate. After measuring sulfate, the remaining anions were tested using the same strategy. In general terms, an incremental TFPB$^-$ concentration results in an improvement of selectivity (i.e., from -3.2 to -4.1 over chloride for M1 and M3 respectively). However, addition of TDMA$^+$ deteriorates the selectivity, obtaining a Hofmeister pattern as was suggested for charged ionophores (the charged ionophore is electrically charged in its uncomplexed form) [23].

Having established the optimum membrane composition the potentiometric system was validated in house for the detection of excretory nitrite in undiluted human urine using M3. The spiked test was performed using undiluted urine of a healthy volunteering individual. Collected samples were spiked with various concentrations of NaNO$_2$ in the range between 10$^{-8}$ to 10$^{-4}$ M and the pH was adjusted to 5 by adding HCl. Fig. 10 shows calibration curves recorded undiluted urine sample. In a background of 200 mM chloride and with the logarithmic selectivity coefficient log $K^{	ext{NO}_2\text{Cl}}_{\text{NO}_2\text{Cl}} = -4.1$, one expects a detection limit for nitrite of ca. 15 μM, while the normal range for nitrite is just 1–10 μM [37]. Despite the complexity of undiluted urine matrix a variation of only one order in the LOD was observed for the intercept and slope, respectively.

4. CONCLUSIONS

A new salophen ionophore [(salph)Co(III)OAc] was synthesized, purified and characterized. This ionophore selectively recognizes nitrite in a lipophilic sensing matrix. The interaction between the ionophore and nitrite is confirmed by two spectroscopic techniques, UV–vis and ATR-IR. As a result of the ionophore–NO$_2^-$ complex, the UV–vis spectrum exhibited a blue shift of 50 nm owing to the change of the electronic structure. The incorporation of nitrite and the subsequent release of acetate are clearly observed using thin membrane films measured by ATR-IR. These two experiments confirmed the double functionality of the ionophore as ion-exchanger and ionophore, which is only found in charged ionophores. Moreover, an apparent formation constant is calculated utilizing the well-established sandwich membrane protocol. This value is somewhat lower (10$^9$) in comparison with typical ionophores for monovalent cations (10$^8$ to 10$^10$). A wide number of potentiometric experiments with different membrane compositions confirm that the presence of cation exchanger enhances the selectivity coefficient by at least one order over chloride. In contrast, anion exchanger sites deteriorate the selectivity coefficient tending to the Hofmeister pattern. Undiluted urine sample spiked with different nitrite levels are quantified in a wide range.

Beyond the observed analytical characteristics of this ionophore, which are essential to the ability of the ionophore to recognize nitrite, this report bring new tools such as ATR to characterize ion-exchange properties in ionophore-based...
membranes. There is much room for improving ionophore based anion-selective membrane and the field is in need for an acceleration of this research direction.

ACKNOWLEDGMENT

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.electacta.2015.03.180.

References


Preparation and Thermoelectric Properties of Chevrel-Phase Cu$_6$Mo$_6$S$_8$ ($2.0 \leq x \leq 4.0$)

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Chevrel-phase sulfides Cu$_6$Mo$_6$S$_8$, where $2.0 \leq x \leq 4.0$, were prepared by reacting appropriate amounts of Cu, Mo, and MoS$_2$ powders at 1273–1523 K for 8 h in vacuum. The samples were then densified by pressure-assisted sintering at 1223–1473 K for 1 h at a pressure of 30 MPa in vacuum. The density of all the sintered samples was greater than 95% of the theoretical density. X-ray analysis showed that all the sintered samples consisted entirely of the hexagonal Chevrel phase. The value of the lattice parameters $a$ and $c$ increased with the Cu content. Measurement of the Seebeck coefficient, electrical resistivity, and thermal conductivity was carried out on single-phase sintered Cu$_6$Mo$_6$S$_8$ samples in the temperature range of 300–950 K. All the sintered samples had a positive Seebeck coefficient. Further, the thermoelectric properties improved when the Cu content was increased. With an increase in the Cu content, the Seebeck coefficient and electrical resistivity increased, while the thermal conductivity decreased. The highest dimensionless thermoelectric figure of merit $ZT$ (0.4) was observed in Cu$_6$Mo$_6$S$_8$ at 950 K. [doi:10.2320/matertrans.MAW200918]

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Keywords: thermoelectric materials, Chevrel phase, pressure-assisted sintering, Seebeck coefficient, electrical resistivity, thermal conductivity, thermoelectric figure of merit

1. Introduction

Thermoelectric devices can directly convert waste heat into electrical energy, and hence, they are expected to play an important role in establishing a sustainable society. For efficient conversion of waste heat to electrical energy, it is important to develop thermoelectric materials with high conversion efficiency. The efficiency of a thermoelectric material is determined by its thermoelectric figure of merit ($ZT$), which is a dimensionless parameter. $ZT$ is given by $S^2T/\kappa$, where $S$, $T$, $\rho$, and $\kappa$ are the Seebeck coefficient, temperature, electrical resistivity, and thermal conductivity, respectively. For the metals or degenerate semiconductors, the magnitude of Seebeck coefficient is inversely proportional to the carrier concentration, and proportional to temperature, and effective mass of the carrier. The electrical resistivity is related to carrier concentration through $1/\rho = ne\mu$, where $n$, $e$, and $\mu$ are the carrier concentration, charge of an electron, and carrier mobility, respectively. The thermal conductivity is the sum of the electronic contribution $\kappa_e$ and the lattice contribution $\kappa_l$, i.e., $\kappa = \kappa_e + \kappa_l$. Moreover, the electronic contribution is directly related to the electrical resistivity through the Wiedemann-Franz law, $\kappa_e = L T/\rho$, where $L$ is the Lorenz number ($2.45 \times 10^{-8}$ W K$^{-2}$).

Ternary molybdenum chalcogenides with the general formula M$_4$Mo$_6$X$_8$ (M = Li, Ti, Cr, Fe, Ni, Cu, Zn, Cd, Sn, Pd, rare-earth metals, etc.; X = S, Se, Te) were first synthesized by Chevrel et al., and therefore, they are referred to as Chevrel phases. Several reviews on the crystal structure and superconductivity of Chevrel phases have been published. The host structure of a Chevrel phase consists of a stacking of Mo$_4$X$_8$ clusters. The M atoms fill the space between the Mo$_4$X$_8$ clusters. While large M atoms such as Pd result in the formation of stoichiometric compounds, small M atoms such as Cu afford nonstoichiometric compounds. For example, it is well-known that the Cu content in Cu$_6$Mo$_6$S$_8$, which has a hexagonal (rhombohedral) structure (space group: $R3$), is in the range of $1.0 \leq x \leq 4.0$.

Recently, Chevrel phases have attracted considerable attention since they are potential high-$ZT$ thermoelectric materials. Most of the previous studies on Chevrel phases have focused on Chevrel-phase selenides. Band structure calculations have been carried out for several Chevrel-phase selenides for predicting their thermoelectric properties. The results suggest that CrMo$_6$Se$_8$, FeMo$_6$Se$_8$, NiMo$_6$Se$_8$, Cu$_2$Mo$_6$Se$_8$, and SnMo$_6$Se$_8$ exhibit metallic behavior, while TiMo$_6$Se$_8$, Zn$_2$Mo$_6$Se$_8$, and Cd$_2$Mo$_6$Se$_8$ behave like semiconductors. Moreover, the results imply that the Fermi level of TiMo$_6$Se$_8$, y shifts to the valence band edge when the Se content decreases. Therefore, the thermoelectric properties of Chevrel phases would be improved by altering their chemical compositions. However, the effect of M content on the thermoelectric properties of M$_4$Mo$_6$Se$_8$ has not yet been investigated in detail.

Caillat et al. measured the Seebeck coefficient, electrical resistivity, and thermal conductivity of M$_4$Mo$_6$Se$_8$ (M = Fe, Ti) and found that $ZT$ of Cu$_{1.38}$Fe$_{0.66}$Mo$_6$Se$_8$ is 0.6 at 1150 K. Since Chevrel-phase chalcogenides have similarity in the electrical and thermal properties, the Cu-filled Chevrel-phase sulfides are also expected to have excellent high-temperature thermoelectric properties.

Tsubota et al. have investigated the thermoelectric properties of Chevrel-phase sulfides M$_4$Mo$_6$S$_8$ (M = Fe, Ni, Ag, Zn, Sn, Pb, Cu). However, X-ray analysis shows that all the aforementioned sulfides contain a large amount of impurity MoS$_2$ phase. The $ZT$ of all the aforementioned sulfides is less than 0.1. In order to confirm whether Chevrel-phase sulfides can be used as thermoelectric materials, it is necessary to investigate the thermoelectric properties of single-phase samples.
The purpose of this study was to prepare single-phase Cu₉Mo₆S₈ samples and study the effect of Cu content on their thermoelectric properties so as to confirm the feasibility of using Chevrel-phase sulfides as thermoelectric materials. The samples were prepared by reacting appropriate amounts of Cu, Mo, and MoS₂ powders at 1273–1523 K and subjecting the resulting product to pressure-assisted sintering. The Seebeck coefficient, electrical resistivity, and thermal conductivity of the sintered samples were measured over the temperature range of 300–950 K.

2. Experimental

The preparation method used in this study is almost the same as that developed by Kondo et al. Commercial Cu powder (purity: 99.99%; particle size: 1 μm; Kojundo Chemical Laboratory Co., Ltd., Japan), Mo powder (purity: 99.9%; particle size: 1.5 μm; Kojundo Chemical Laboratory Co., Ltd., Japan), and MoS₂ powder (purity: 99.9%; Kojundo Chemical Laboratory Co., Ltd., Japan) were used as the starting materials. Appropriate amounts of these powders were mixed well and placed in boron nitride crucibles. The starting materials were evacuated to 1273–1523 K at a rate of 5 K/min and maintained at this temperature range for 8 h. The samples were then gradually cooled to room temperature.

The samples were consolidated by pressure-assisted sintering for the fabrication of dense compacts. The powders prepared as mentioned above were placed into graphite dies. The dies were set in a pulsed electric current sintering apparatus (SPS-515S, SPS Syntex Inc., Japan). The chamber of the apparatus was then evacuated to 7.0 × 10⁻³ Pa. The samples were heated to 1273–1523 K at a rate of 5 K/min and maintained at this temperature range for 8 h. The samples were then gradually cooled to room temperature.

The crystal phase in the sintered samples was studied by powder X-ray diffractionmetry (XRD) using Cu Kα radiation (Rint-Ultima+, Rigaku Co., Japan). The density of each sintered sample was measured using the Archimedes method (AUX120-SMK401, Shimadzu Co., Japan). The microstructure of the fractured surfaces and the polished and corroded surfaces was observed under a scanning electron microscope (SEM; JSM-6301F, JEOL Ltd., Japan) at 20 kV accelerating voltage.

The Seebeck voltage and electrical resistivity of the sintered samples were measured simultaneously (ZEM-3, ULVAC-RIKO, Inc., Japan); in this method, the thermal conductivity was calculated from the density, heat capacity, and thermal diffusivity.

3. Results and Discussion

Figure 1 shows the XRD patterns of the sintered Cu₉Mo₆S₈ samples. These XRD patterns are in good agreement with those previously published, thereby showing that all the prepared samples consist entirely of single-phase Cu₉Mo₆S₈. No impurity phases are formed in any of the samples. The change in the Cu content results in the changes in the atomic positional parameters, and hence the changes in the XRD peak intensities and positions. As shown in Fig. 2, the value of the hexagonal lattice parameters a and c increases with the Cu content. The values of a and c also agree well with those reported.

The density of all the sintered samples is found to be greater than 95% of the theoretical density. As shown in Fig. 3, SEM examination of the fractured surfaces reveals...
that the samples have a dense structure, which is consistent with the results of density measurements. The fracture surface of Chevrel-phase sulfides has been conventionally used for the grain size measurement. From Fig. 3, the grain size of the sintered samples is estimated to be of the order of micrometers. Similarly, in polished and corroded surfaces, the grain size is estimated to be of the order of micrometers. Since the grain size is much larger than the mean free path of electron and phonon, the grain boundaries have no effect on the thermoelectric properties.

Since the sintered samples consist of single-phase Cu$_x$Mo$_6$S$_8$ and have nearly full density, they can be used for investigating the thermoelectric properties of Chevrel-phase sulfides. The temperature dependence of the Seebeck coefficient and electrical resistivity are shown in Figs. 4 and 5, respectively. All the samples have a positive Seebeck coefficient, indicating that the dominant carriers are holes. Moreover, the Seebeck coefficient and electrical resistivity show metallic temperature dependence, i.e., they increase linearly with temperature. It is worth mentioning that the chemical composition of the samples has a remarkable effect on the Seebeck coefficient and electrical resistivity—an increase in the Cu content results in increases in both the Seebeck coefficient and electrical resistivity. These increases in the Seebeck coefficient and electrical resistivity are mainly due to a decrease in the carrier concentration. The electrical properties of the Chevrel phase can be explained in terms of the cluster-valence-electron count (cluster-VEC). The cluster-VEC of M$_x$Mo$_6$S$_8$ is calculated as follows: the number of electrons required to fill the octets of the S atoms is subtracted from the sum of the number of valence electrons of the M and Mo atoms; the resulting value is then divided by the number of Mo atoms. In the case of Cu$_x$Mo$_6$S$_8$, the increase in the Cu content results in an increase in the cluster-VEC and a resultant decrease in the carrier concentration.

As shown in Fig. 6, the thermal conductivity of Cu$_x$Mo$_6$S$_8$ is relatively low, ranging from 1.3 to 3.9 W K$^{-1}$ m$^{-1}$, and increases with temperature. Since the electrical resistivity increases with the Cu content, the electronic contribution decreases with an increase in the Cu content. On the other hand, the lattice contribution remains essentially unchanged when the Cu content is increased and ranges from 0.9 to 1.1 W K$^{-1}$ m$^{-1}$ at 950 K.

Figure 7 shows the temperature dependence of the thermal conductivity, electronic contribution, and lattice contribution for Cu$_{4.0}$Mo$_6$S$_8$. The electronic contribution is higher than the lattice contribution over the entire temperature range considered in this study. The lattice contribution of Cu$_{4.0}$Mo$_6$S$_8$ is very low and increases with temperature. This low value of the lattice contribution is most probably
due to phonon scattering by the Cu vacancies. There are twelve Cu sites in each Mo$_6$S$_8$ cluster in Cu$_x$Mo$_6$S$_8$. However, the upper occupancy limit has been experimentally found to correspond $x \approx 4.0$. Therefore, there are numerous vacancies at the Cu sites. These vacancies are most likely to cause disorder at the Cu sites and thus reduce the lattice contribution.

The $ZT$ values calculated from the measured values of the Seebeck coefficient, electrical resistivity, and thermal conductivity are shown in Fig. 8. From the figure, it is evident that the $ZT$ value of all the samples increases with temperature. It is also to be noted that the $ZT$ value shows a significant increase with the Cu content. The highest $ZT$ (0.4) is observed for Cu$_{4.0}$Mo$_6$S$_8$ at 950 K. On the basis of these observations, it is concluded that Chevrel-phase sulfides are suitable candidate materials for high-temperature thermoelectric devices.

Acknowledgements

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REFERENCES

10) T. Caillat and J.-P. Fleurial: Proc. 16th Int. Conf. on Thermoelectrics,
Preparation and Thermoelectric Properties of Chevrel-Phase Cu$_{x}$Mo$_6$S$_8$ (2.0 ≤ x ≤ 4.0)
Postmodernism Spreading within Scientific Institutions

This idea is especially worrying at the NAS because at least one member of its ‘Committee on Gene Drive’ has written elsewhere that ‘upstream engagement tends to push dialogue towards issues of value and ideology and in such cases there is much less willingness on the part of protagonists to reconsider evidence on the basis of its scientific merit’ [8]. In the same article, J. Tait accurately analyzed that STS sociology ‘challenged the authority of science, particularly its presumed impartiality and its role as provider of public benefits . . . and sought to change the political landscape, again towards greater public participation’. Further, it sought to ‘reshape . . . the very foundations on which the scientific enterprise rests’. In this context, the NAS report recommendation to ‘align research and policy with public values’ and to consider it as ‘particularly important for ecological risk assessment’ is simply a delusion.

The report ambiguously fluctuates in its definition of what ‘engaging the public’ actually means. It is viewed either as being open to dialogue (a value of Enlightenment) in relation to the idea that ‘technical expertise is insufficient for ensuring good governance’ (which is obvious) or even to ‘the recognition of the contributions of local understanding to the practice of science’ (which all depends what ‘local understanding’ entails). The latter assumption is allegedly illustrated by examples provided exclusively by again the same school of sociology. One publication [9] criticizes the lack of a ‘reflexive capability’ of scientists regarding the radioactive fall-out from Chernobyl experienced by sheep-farmers in England, stating ‘that scientific knowledge tacitly imports and imposes particular and problematic versions of social relationships and identities’. This criticism represents an extension to science of the postmodern ‘tyranny of guilt’ [10]: scientists should feel guilty for being ‘imperialistic’ and for defending their own identity (at the expense of others). Another publication claims that scientific knowledge is shaped by struggle for power and by controversy [11]. The example cited is that of AIDS research, described as a social and political phenomenon because the AIDS movement is considered as having transformed biomedical research practices. In my humble opinion, the identification of the AIDS causal agent owes more to the expertise of virologists and molecular biologists (who probably had limited ‘reflexive capability’).

Why Postmodernism as an Assault on Science Has Been Difficult to Grasp by Scientists

Postmodernism is often confused with values of respect and democracy [2]. However, science is not a matter of democracy – it is about the application of a method, and it is an elitist activity, open to all provided that one learns and applies the scientific method. Scientists should be able to ‘reflect upon and revise their own opinion’ without injunction from postmodern political correctness.

References

Antimicrobials; bacterial conditions includes the reduce/abolish Conditioning film

Figure 1. Schematic Illustration of a Ureteral Stent in the Urinary Tract Environment. This environment includes the abiotic material (red), biotic surfaces (orange), environmental conditions (blue), and microbiota (green). The biological surface is composed of epithelial cells, which secret host defense factors (A). To protect the abiotic surface against colonization, either anti-adhesive (E) or biocidal (C) modifications can be introduced. Conditioning film formation can change the release profile of antimicrobials and, together with dead cells, can reduce/abolish the functionality of active coatings (D). Furthermore, the successful prevention of surface colonization is highly dependent on microbial surface properties (E), which are affected by their environmental conditions (F). Bacterial cells in biofilms are embedded in a self-produced EPS matrix that impedes penetration of the antimicrobials (G). Key: ——— glycosaminoglycan (GAG); — live bacterial cells; — dead bacterial cells; — extracellular polymeric substances (EPS); — antimicrobial peptides; conditioning film.

Of 11282 patients had at least one healthcare-associated infection. More than a quarter of these infections were related to medical devices [1]. Concerns are growing because of the increasing occurrence of bacterial resistance against currently available antibiotics. Particularly when living as biofilms, bacteria can tolerate up to 1000-fold higher concentrations of antibiotics compared to planktonic cells [2]. Severe device-associated infections often require complete replacement of the corresponding medical device, which is associated with high risks for patients and high social costs [3]. Even though many antimicrobial materials with anti-adhesive, contact-killing, drug-release, or immune-stimulating properties have been suggested for use in medical devices, only a handful have made the translation to clinical practice [4]. One of the reasons for this failure is related to the lack of relevant biological in vitro biofilm models that allow the in vivo antimicrobial and anti-biofilm performance of the device to be predicted. Such a laboratory “biofilm model” simulates the natural (in vivo) situation with a focus on selected relevant factors such as materials, fluid flow, growth media (nutrients), and intercellular interactions. For example, the developed material surfaces can be covered by a conditioning film derived from hosts (e.g., extracellular polymers and cellular debris) and/or dead bacterial cells, which alters the surface properties, and may allow bacterial adhesion and biofilm formation [5]. Thus, the conditioning film needs to be considered. To date, possibly owing to a lack of awareness of the complexity of biological systems, or difficulties in mimicking them, many studies have applied irrelevant cultivation conditions or have used inappropriate microbial species or strains.

Based on the example of catheter-associated urinary tract infections (CAUTIs), which are among the most common healthcare-associated infections [6], we provide a short overview of the important factors affecting the interaction of
microbes with the inserted catheters/stents, and we highlight their relevance for the design and development of predictive in vitro biofilm models (Figure 1). Such predictive in vitro models would help in developing biomaterials that are less prone to bacterial colonization, reducing the occurrence of device-related infections.

**Limitations of the In Vitro Biofilm Models Reported to Date**

One pitfall in designing a biofilm model for antimicrobial testing lies in using relevant bacterial strains. In many studies, irrelevant bacterial strains or cultivation conditions have been used, and the suitability of many published promising antimicrobial substances and materials for in vivo applications therefore needs to be questioned. For example, despite their great merit for studying mechanisms of attachment/biofilm formation, mutated bacterial laboratory strains with reduced abilities to attach to surfaces have instead been used to assess the antimicrobial activity of clinically-relevant materials. The mutations, for example fimbral mutants of Escherichia coli, reduce the ability of the cells to form biofilms, which in turn may increase their susceptibility to antimicrobials [2].

In both environmental and medical settings, bacteria usually grow under limitations of specific nutrients and under the selection pressure of host defense mechanisms. Nutrient limitations can lead to an altered gene expression profile, increasing the expression of virulence factors and biofilm formation. Consequently, a growth medium that is artificially rich in nutrients may lead to an irrelevant phenotype, making bacteria more susceptible to antimicrobials. Furthermore, biofilms formed under static growth conditions (i.e., suspension cultures) can have different gene expression patterns from those formed under continuous medium flow as a result of different shear stresses [7]. Therefore, results from static growth assessment should be counterchecked with flow conditions, if relevant in vivo.

**How Close to Reality Can a Biofilm Model Be?**

Ideally, an in vitro biofilm model can help to predict the performance of novel antimicrobials or antimicrobial materials in vivo. Designing such a model requires a detailed consideration of the biological setting in vivo (i.e., location where the biomaterial is inserted or biofilms are formed). Despite all efforts to mimic the in vivo situation, the model needs to be reduced to the most important and inevitable factors that may affect the antimicrobial activity. These include bacteria-derived, host-derived, and abiotic factors (Figure 2).

The choice of the bacterial strains to be used depends on the targeted biological setting as well as on the duration of the testing. Selecting appropriate strains requires knowledge of the microbial consortium relevant for the specific device application. However, although data on viable but non-cultivable bacteria are available, only cultivable strains can be used to generate in vitro biofilm models. Furthermore, it is difficult to simulate the endogenous, healthy microbial flora and its interaction with potentially pathogenic strains in the context of a medical device-specific growth environment.

As mentioned above, growth media impose a further challenge in establishing an in vitro biofilm model. In the case of urinary catheters and stents, crystalline biofilms are often formed as a consequence of increased urine pH and the precipitation of phosphate salts caused by urease-secreting bacteria [8]. Various studies have used genuine human urine for bacterial cultivation, leading to the problem of batch-to-batch variation. Alternatively, artificial urine may mimic passivation and inactivation of (antimicrobial) surfaces through the precipitation of such crystals (e.g., struvite and hydroxyapatite). The frequently used artificial urine formulation [9], however, contains substantial amounts of yeast extract, leading to an unrealistically high nutrient supply. Further improvements included replacement of yeast extract by a trace element solution [10]. However, the central role of iron limitation in the urinary tract, part of the endogenous antibacterial defense, has mostly been neglected. Therefore, the presence of iron in individual media components should be taken into consideration.

A remaining challenge in this context is that human urine contains relatively high...
amounts of antimicrobial proteins and peptides (e.g., uromodulin, RNase 7, and antimicrobial peptides), particularly as a consequence of urinary tract inflammation (e.g., [11]). Including such factors into a biofilm model, however, is difficult because of high costs and challenges in the purification of such peptides and proteins. In addition, factors such as temperature, shear stress (i.e., flow vs static conditions), and osmolality can influence the outcome of an in vitro biofilm study. In some settings, continuous medium flow is closer to reality compared to static growth conditions because, in many cases, antimicrobials can elute in a single burst of release, with a subsequent loss of functionality of the bioactive coating.

In a biological environment, (antimicrobial) devices are in contact with biotic interphases, such as epithelial cells and/or biological fluids. Extracellular polymeric substances (EPS), whether host- or bacteria-derived, as well as cellular debris, can compromise the performance of antimicrobials by acting as scavengers [12]. These EPS and cell debris can also cover the antimicrobial surfaces, increasing bacterial attachment and shielding the bacteria. In some studies, small percentages of serum are added to the medium, which is a step in the right direction [13].

Even higher complexity can be added to a biofilm model once the interactions with the host cells are taken into account. For example, an in vitro model including bacteria, osteoblast-like cells, and macrophages for bone-implant testing has been reported to possess the features concurring with clinical observations [14]. Damage of the host cells by the presence of biomaterials may play a key role in susceptibility to bacterial infections. In the urinary tract, cell surfaces are covered by a mucoid layer of glycoproteins (i.e., the uropilins) that reduce friction forces and serve as an antimicrobial barrier. The presence of medical devices such as ureteral stents or catheters may compromise the cells, particularly when an abrasive crystalline biofilm has formed on the medical device. As a consequence, bacteria may attach to and invade the epithelial cells, thereby forming intracellular bacterial communities that are not recognized by the immune system and are difficult to treat with antibiotics [15]. Hence, prokaryotic/eukaryotic coculture biofilm models would provide deep insights into the antimicrobial performance of materials, but such models are inherently associated with major challenges. The above-presented challenges encountered in in vitro biofilm models for studying urinary catheter/stent materials demonstrate the difficulty of including all relevant factors and the need for an appropriate selection of parameters that are relevant to the biological setting of interest.

Concluding Remarks

Even though many of the commonly employed in vitro biofilm models provide valuable insights into biofilm behavior on a given surface, it must be recognized that each model is limited owing to the use of specific bacteria under specific environmental conditions. Thus, when evaluating data generated by such models, it is crucial to consider the clinical relevance of the model. Consideration of cellular interactions, for example with commensal bacteria and/or human cells, would potentiate the relevance of novel and advanced in vitro models.

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References


Science & Society

Can We Get Rid of Palm Oil?

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Concerns over the sustainability of palm oil have triggered debates about its role in a bio-based economy, but can we get rid of it? Although the quick answer is no, we should eliminate unsustainable land-use practices. However, currently, technical and financial support for land-users to adopt sustainable land-use practices in the cultivation of palm oil is largely missing.

Why No Palm Oil?

Concerns over the sustainability of palm oil have triggered debates about its role in a bio-based economy that aims to
Automated Synthesis of Monodisperse Oligomers, Featuring Sequence Control and Tailored Functionalization

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Supporting Information

ABSTRACT: Long, multifunctional sequence-defined oligomers were obtained on solid support from a protecting-group-free two-step iterative protocol, based on the inherent reactivity of a readily available molecule containing an isocyanate and a thiolactone. Aminolysis of the latter entity with an amino alcohol liberates a thiol that reacts with an acrylate or acrylamide, present in the same medium. Subsequently, a new thiolactone can be reinstated by means of an α-isocyanato-γ-thiolactone. Different acrylic compounds were used to incorporate diverse functionalities in the oligomers, which were built up to the level of decamers. The reaction conditions were closely monitored in order to fine-tune the applied strategy as well as facilitate the translation to an automated protocol.

Polymer chemists have made great progress in the last decades in the preparation of functionalized and complex polymer architectures with defined structure–property relationships, yet these structures do not reach the same potential as their natural counterparts (DNA, RNA and proteins). As a result, in the past decade, efforts have increased to achieve control over the primary structure of macromolecules in hopes of creating a stronger and/or unique correlation between properties and structure. Many different methods have been explored for the synthesis of such sequence-controlled macromolecules. (Semi)automated protocols, which facilitate iterative solid phase synthesis (SPS) of oligonucleotides and oligopept(o)ides, have gained much attention for the preparation of defined/control sequences. SPS revolutionized the fields of peptide and oligonucleotide chemistry. This versatile method enabled the fast synthesis and isolation of oligomers. The efficient automation of SPS allowed even faster synthesis and parallel approaches to build a wide variety of natural oligomers, nowadays even up to a ton scale. Automated synthesis of oligopeptoids has been achieved via an efficient submonomer strategy, which does not require any protecting groups. Given these successes, adapted protocols have been employed for the synthesis of sequence-defined polymers using phosphoramidite chemistry, which is classically used for the synthesis of poly- and oligonucleotides on DNA synthesizers.

Lutz et al. applied this chemistry to make sequences with high DP (>100), using a limited monomer alphabet consisting of two custom-made monomers, with the aim to produce binary code type (1/0) sequences.

This paper aims to present a robust and versatile protocol for the synthesis of sequence-defined multifunctional oligomers based on thiolactone (Tla) chemistry and its successful automation. Recently, we have shown that immobilizing a Tla unit on a solid support enables chain extension after on-resin aminolysis. In this previous submonomer strategy, the monomer alphabet, encompassing amines and thiolactone units, is broad and protecting groups are not necessary. However, the reported approach was limited to the synthesis on very small scale of tetramers with moderate crude purity. As a result of disulfide formation during on-resin aminolysis, treatment with phosphines as reducing agents was necessary, promoting the accumulation of side products. Therefore, to allow for the preparation of much longer, highly functional sequences, with the additional possibility to translate it to an automated protocol, a new protecting-group-free strategy was developed. Remarkable features are much higher versatility in side chain functionality and backbone composition, and elevated crude purity.

The implementation of the nucleophilic amine-thiol-ene conjugation is a breakthrough development for Tla-based SPS. In the first step of the synthetic cycle (STEP 1, Scheme 1), the immobilized Tla is selectively opened through the primary amine function of an amino alcohol (ethanolamine or 4-amino-1-butanol), releasing a thiol that subsequently reacts with an acrylate or acrylamide through a nucleophilic thiol–ene reaction. A wide set of functionalities can be introduced by using abundantly available acrylics whereas the nature of the backbone can be varied through the selected amino alcohol. Moreover, it is anticipated that disulfide formation can be completely avoided in this mild additive-free approach. In the next step, chain extension is performed via the reaction of the readily available and stable α-isocyanato-γ-Tla (Scheme S2) with the remaining alcohol function (STEP 2, Scheme 1).

This two-step protocol can be repeated until a unique oligomer with targeted length, sequence and functionality is obtained. In the present study, the prepared structures feature amides and urethanes in the backbone and ester- and amide-linked side chains. The α- and ω-end groups of these sequences depend on two parameters: the functional group needed to initially immobilize the Tla moiety on the solid phase (α-terminus) and the stage at which the iterative protocol is terminated at the other extremity (ω-terminus). There are two possibilities as far as the latter is concerned. The sequence can be terminated after step 1, which results in an alcohol, or after step 2, which results in a Tla. The nature of the α-end group, on the
other hand, depends on the functionality used to connect the Tla on the cross-linked polystyrene resin. In addition to a Tla-containing acid\textsuperscript{33} as linker, a Tla-containing alcohol was evaluated. The loading of the hydroxyl-functionalized Tla linker was 0.85 mmol Tla units per gram resin (53\% loading efficiency, Scheme S1 and Figure S4).\textsuperscript{36}

The reaction conditions were carefully investigated to prepare sequence-defined oligomers efficiently and to transfer subsequently this protocol to an automated approach. Optimization parameters included the reaction stoichiometry, the choice of solvent and the reaction times used for each step. The amine/acrylate ratio used in the first step is important, as this influences the extent of disulfide formation. A ratio of one to two was found to prevent this side reaction. Next, the chosen solvent must promote the reaction between the three reaction partners as well as ensure the swelling of the resin. Although DMF and CH$_2$Cl$_2$ are commonly applied in SPS, they would cause difficulties in the present protocol. Indeed, DMF promotes the side reaction between an acrylate and an amine,\textsuperscript{37} while we reported earlier that CH$_2$Cl$_2$ can react with the thiol formed after aminolysis.\textsuperscript{38} In CHCl$_3$, the occurrence of aza-Michael addition was found to be less problematic. The last important parameter for Step 1 is reaction time. A series of measurements demonstrated that the reaction is completed after 15 min (Figure S5). However, the first step is performed twice to ensure full conversion for longer sequences.

The second step of the iterative protocol consists of the reaction between the $\alpha$-isocyanato-$\gamma$-Tla and an alcohol incorporated in the first step via aminolysis of the Tla. The reaction is performed with 10 equiv of the isocyanate and 0.025 equiv of dibutyltin dilaurate (DBTL). Subsequently, three dry solvents were tested: DMF, ethyl acetate and CHCl$_3$ (Figures S6–S8). This screening revealed that the reaction is completed within 30 min when using CHCl$_3$, after 2 h for ethyl acetate and still not finished after 8 h for DMF. In the optimized protocol, the chain extension in CHCl$_3$ is, however, made to last 1 h to ensure full conversion.

Subsequently, the protocol was closely followed while first making a trimer sequence using benzyl, methyl and tetrahydrofurfuryl acrylate (C$_0$5–C$_3$5, C$_x$ = performed cycles). HR-MS, LC-ESI-MS and NMR spectra were recorded to verify sample purity and incorporation of side chain residues (Tables S1–S5 and Figures S9–S16). The LC traces of crude reaction mixtures consistently displayed one major peak that corresponded with the expected product, demonstrating that the products display no deletions (Figure 2A) and that 5\% of the hydroxyl end group is transformed into the corresponding TFA ester (Figures S9 and S11). Next, two extra functionalities were incorporated using butyl and isobornyl acrylate, yielding a highly pure pentamer (C$_5$) (Figure 1, Figures S17–S18 and Table S6). Continuing with this pentamer, a heptamer (C$_7$) was made by incorporating

\textsuperscript{36}The immobilized thiolactone group is opened with an amino alcohol and subsequently reacted with a functional acrylate or acrylamide (STEP 1) whereas $\alpha$-isocyanato-$\gamma$-Tla serves to reinstate the Tla moiety (STEP 2).

Figure 1. Overview of different sequences, illustrating the diversity of incorporated acrylics. CS and EE3 were made from ethanolamine and various acrylates, while BUTOL1 and BUTOL2 are examples of a pentamer and a trimer in which 4-amino-1-butanol is incorporated in the backbone. For BUTOL2, there is also an acrylamide incorporated in the side chain.
additional benzyl and tetrahydrofurfuryl residues (Figures S19−S20 and Table S7).

Next, five decamers were prepared, the first one (C10) by starting from the heptamer with the incorporation of methyl, butyl and isobornyl groups. The second (T10) one was a homodecamer with only tetrahydrofurfuryl groups as functionality. The third (BST5) and fourth ((BT)5) were built with the latter acrylate and benzyl acrylate to make two decamers, respectively consisting of two pentamer blocks and an alternating structure. The small retention time difference in the LC traces of both decamers originates from their different behavior toward the column (Figure 2B). Finally, the fifth decamer had a sequence similar to the first one, but the acid linker was applied to incorporate a different α-end group (Figures S21−S30 and Tables S8−S12).

To emphasize the almost unlimited choice for side chain functionalities, two trimers (EE3 and DMAE3) were made that contained an n-butyl group, an acid and a tertiary amine in the side chains (Figure 1). The acid and amine functionalities could be incorporated by using respectively 1-ethoxyethyl and 2-(dimethylamino)ethyl acrylate.39 Acid cleavage from the resin also removed the ethoxyethyl group, resulting in an acid residue. To extend further the functionality scope from acrylates to acrylamides, N,N-diethylacrylamide was also incorporated in a trimer (DEAA3) (Figures S31−S36 and Tables S13−S15).

Finally, to illustrate the high versatility, also the backbone has been modified by using 4-amino-1-butanol instead of ethanolamine. In previous research,35 the effect of the amine on the kinetics of aminolysis was investigated and taken into account in the protocol (Figure S37). Although the first synthesis of a trimer indicated a deletion (Figure S38), the protocol was modified for this specific aminolysis to four times 15 min. With this adjustment, a high purity pentamer (BUTOL1) was made with only tetrahydrofurfuryl groups as side chain functionalities and a trimer (BUTOL2) with three side chain functionalities and based on two alcohol amines (Figure 1, Figures S39−S42 and Tables S16−S17).

The next target was the automatization of the protocol to facilitate the iterative work and synthesize several sequences in parallel, thus significantly reducing the overall process time. To achieve this goal, a peptide synthesizer was adapted to our needs and all sequences were prepared in a single nonstop run. In comparison with a standard synthesizer, extra bottles were installed for washing solvents and closed vessels were required for the inert storage of the α-isocyanato-γ-Tla, DBTL and dried CHCl₃. This last adaptation was absolutely necessary because of the water-sensitive urethane synthesis during each second step of the protocol (see movie and picture with additional information in SI).

First, a trimer (R1) was made to adjust instrument settings (Figure S31). Next, six random hexamers (R2-R7) were synthesized in parallel using benzyl, butyl and tetrahydrofurfuryl acrylate. This second screening was equally very effective, since every hexamer was characterized by a high level of purity (Figures S32−S37). A nonamer (R8) was also prepared with a high abundance, but traces of deletions could be noticed (Figure S38).

The final test was a comparison between (BT)5 decamers, made in a manual and in an automated way (R9) (Figure 3).

The LC trace of the robot-made decamer indicated that the decamer was formed and displayed the highest abundance, but it
also shows the presence of a nonamer (#), missing a benzyl functionality, and a decamer without the presence of a Tla unit (*). In comparison with the storage vessels, the reactors for the automated synthesis are open, which can cause problems during the chain extension, because isocyanates are sensitive to humidity in the air. The formation of a white precipitate, which corresponds to dithiolactone urea (Figures S40–S41), can confirm this air sensitivity. On the other hand, it should be emphasized that the time for the synthesis of a decamer is obviously much shorter with the automated protocol (±33 h versus 3 to 5 days).

In conclusion, a robust chemical platform was developed to perform the protecting-group-free SPS of multifunctional sequence-defined oligomers using Tla chemistry. Various functional groups could be incorporated by means of acrylics during aminolysis. Furthermore, both the nature of the backbone and the end-groups at both termini has been adapted. Functionalized oligomers, up to decamers, could be prepared with high purity. Finally, the protocol could be efficiently translated to an automated approach on an adapted peptide synthesizer for an accelerated synthesis of libraries of sequence-defined oligomers.

In the near future, we hope to be able to show that the available racemic structures can be applied in the field of molecular data storage. The option to store information both in the multifunctional side and/or main chains can result in a quite significant progress in terms of data capacity on a single oligomer. Future work will also focus on the stereocontrol of the applied thiolactone unit, because this is expected to have an impact on the folding behavior of the sequence-defined structures.46

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b07120.

Experimental procedures; synthesis of different Tla’s and sequences; kinetic study; NMR and LC-ESI-MS data (PDF)
Adapted peptide synthesizer setup (AVI)

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REFERENCES
(40) Zuckermann, R. N. Biopolymers 2011, 96, 545.
Since Bogdanovic and Schwickardi illustrated the possibility of reversibly storing hydrogen in sodium alanate, extensive research efforts have been dedicated to investigating the hydrogen storage potential of complex metal hydrides. In particular, borohydrides have attracted great interest because of their superior gravimetric hydrogen content. Of these, magnesium borohydride Mg(BH$_4$)$_2$, first reported in 1950 and more recently studied for hydrogen storage, has attracted attention because of its relatively low hydrogen-release temperature and reversibility. Furthermore, borohydrides are strong reducing agents that are widely used in organic and inorganic syntheses. This reducing power translates to high inorganic syntheses. This reducing power translates to high stability against electrochemical reduction; this stability could be exploited in highly reductive environments, such as battery anodes. Therefore, for the first time, we have conducted research towards harnessing this property of borohydrides for their use in rechargeable batteries. In particular, we have been focusing on utilizing a Mg(BH$_4$)$_2$ based electrolyte in a rechargeable magnesium battery.

Recently, magnesium batteries have received increased attention as alternatives to the lithium-based battery because of the high volumetric capacity (3832 mAh cm$^{-3}$), improved safety (nondendritic), and abundance of Mg metal. Despite the potential of Mg batteries, several key challenges need to be overcome for this technology to become viable. For instance, current state-of-the-art electrolytes use organomagnesium salts and complexes as they are the only ones known to be compatible with the Mg anode that allow for reversible electrochemical Mg deposition and stripping. Although some of these electrolytes have shown impressive stability against electrochemical oxidation, they were also found to be corrosive. This property was attributed to the presence of chlorides in either/both their cations and anions. Conventional inorganic and ionic salts such as Mg(ClO$_4$)$_2$ were found to be incompatible with the Mg anode as a result of the formation of an ion-blocking layer formed by their electrochemical reduction. Hence, the discovery of halide-free electrolytes with high reductive stabilities is crucial for realizing a practical rechargeable Mg battery system.

Herein, we propose a new class of electrolytes based on Mg(BH$_4$)$_2$ for a Mg battery. We show the first example of electrochemical reversible Mg deposition/stripping in a halide-free inorganic salt in both tetrahydrofuran (THF) and dimethoxyethane (DME) solvents. An increase of several orders of magnitude in the current densities, and high coulombic efficiencies of up to 94% are observed in DME when LiBH$_4$ is used as an additive. Furthermore, we use this electrolyte in a rechargeable Mg battery, thus giving the first example of a borohydride electrolyte in a battery system. This work also illustrates the unique properties of borohydrides and opens the door for designing a whole new class of electrolytes for Mg batteries.

Mg deposition/stripping was studied for Mg(BH$_4$)$_2$ in ether solvents. Figure 1a shows the cyclic voltammogram obtained for 0.5 M Mg(BH$_4$)$_2$/THF where a reversible reduction–oxidation process took place with onsets at −0.6 V/0.2 V and a 40% coulombic efficiency (Figure 1a, inset), thus indicating reversible Mg deposition and stripping. X-ray diffraction (XRD) confirmed that the deposited product from the galvanostatic reduction of the above solution (Figure 1b) was hexagonal Mg, thereby establishing the compatibility of Mg(BH$_4$)$_2$ with Mg metal. The electrochemical oxidative stabilities measured on platinum, stainless steel, and glassy carbon electrodes were 1.7, 2.2, and 2.3 V, respectively (Figure S7). These results showed that for the first time: 1) Mg(BH$_4$)$_2$ is electrochemically active in THF, that is, ionic conduction is possible, and 2) reversible magnesium deposition/stripping from an inorganic, relatively ionic (Mg Bader charge is +1.67) and halide-free salt is feasible. Although these results are promising, to make this electrolyte more practical for use in batteries the electrochemical performance needs to be improved by lowering the overpotentials, and achieving higher current density and coulombic efficiency. In addition, the demonstration of this performance in less-volatile solvents would make Mg(BH$_4$)$_2$ based electrolytes even more practical. Therefore, DME was selected (its boiling temperature is 19°C higher than that of THF) for further investigations. The cyclic voltammogram obtained for 0.1 M Mg(BH$_4$)$_2$/DME is shown in Figure 1c where a substantial improvement in the electrochemical performance compared to Mg(BH$_4$)$_2$/THF was evident from: 1) a 10-fold increase in the current density, 2) a reduction in the overpotentials (deposition/stripping onsets at −0.34 V/0.03 V versus −0.6 V/0.2 V in THF), and 3) a higher coulombic efficiency of 67% (40% in THF). These findings suggested that the Mg...
electroactive species was present in higher concentration and had increased mobility in DME despite the lower solubility of Mg(BH$_4$)$_2$ in DME versus THF. These results demonstrated that for the Mg(BH$_4$)$_2$ electrolyte, the electrochemical performance in DME is higher than that in THF. In contrast, organomagnesium electrolytes show an optimum electrochemical performance in THF.

To further improve the electrochemical performance, it was pertinent to characterize the electroactive species in Mg(BH$_4$)$_2$ solutions. Therefore, IR and NMR spectroscopic analyses (Figure 2) were conducted for 0.5 M Mg(BH$_4$)$_2$/THF and 0.1 M Mg(BH$_4$)$_2$/DME. The IR B/C$_0$H stretching region (2000–2500 cm$^{-1}$) showed two strong widely separated bands (Mg(BH$_4$)$_2$/THF: 2379 cm$^{-1}$, 2176 cm$^{-1}$ and Mg(BH$_4$)$_2$/DME: 2372 cm$^{-1}$, 2175 cm$^{-1}$); note that the spectra for 0.1 and 0.5 M of Mg(BH$_4$)$_2$ in THF are similar (Figure S2). These IR spectra are similar to those of covalent borohydrides[8] and those of Mg(BH$_4$)$_2$ solvates from THF and diethyl ether[9] where two hydrogen atoms in BH$_4^-$ are forming a bridge to one metal atom (μ bonding). Therefore, we assigned the bands at the higher and lower B/C$_0$H frequencies to terminal and bridging B/C$_0$H vibrations (B/C$_0$H$_t$ and B/C$_0$H$_b$), respectively. The band and shoulder at 2304 and 2240 cm$^{-1}$ were assigned to asymmetric B–H and B–H$_b$ vibrations, respectively. As complete dissociation of Mg(BH$_4$)$_2$ into discreet ions is unlikely (as other borohydrides are in ethers)[10], we propose that Mg(BH$_4$)$_2$ is present as the contact ion pair Mg[(μ-H)$_2$BH$_2$]$^+$, which partially dissociates into [Mg[(μ-H)$_2$BH$_2$]$^+$ and BH$_4^-$ as in [Eq. (1)]; since the different B–H bands most likely overlap, it is not possible to distinguish all the species:

\[
\text{Mg}_{[(\mu-H)_{2}BH_{2}]}^{+} \rightarrow \text{Mg}_{[(\mu-H)_{2}BH_{2}]}^{+} + \text{BH}_{4}^{-}
\]  

(1)

Where [Mg[(μ-H)$_2$BH$_2$]$^+$ may further dissociate:

\[
[Mg[(\mu-H)_{2}BH_{2}]]^{+} \rightarrow \text{Mg}^{2+} + \text{BH}_{4}^{-}
\]  

(2)

For the spectrum of Mg(BH$_4$)$_2$/DME, although the main features present in the spectrum of Mg(BH$_4$)$_2$/THF were retained, the νB–H band is broader and shifted to a lower value and the νB–H$_b$ intensity is relatively weaker. Although νB–H band broadening suggests a pronounced presence of a species similar to that found in THF, the shift in the band maximum indicates a more-ionic B–H bond (the νB–H$_t$ shift is similar to those resulting from BH$_4^-$ ions that have enhanced ionic character, such as in stabilized covalent borohydrides).[8] In addition, the relative weakening in νB–H$_b$ intensity suggests that there is more free BH$_4^-$. The NMR spectrum of BH$_4^-$ in DME (Figure 2b and c) indicates that there is increased boron shielding as the associated signal is shifted by about 0.5 ppm (quintet in $^{11}$B NMR spectrum), and slightly reduced proton shielding (0.01 ppm, quartet in $^1$H NMR spectrum); these results are consistent with B–H bonds that have a higher ionic character than those in BH$_4^-$ in THF (distinguishing B–H$_t$ from B–H$_b$ is not possible likely...
because of rapid hydrogen exchange). These findings are evidence of weaker interactions between Mg$^{2+}$ and BH$_4^-$ within the ion pair and an enhanced dissociation in DME [Eq. (1) and (2)]. So despite the fact that DME has a slightly lower dielectric constant (7.2) compared to THF (7.4), its chelation properties (owing to the presence of two oxygen sites per molecule)\cite{11} resulted in an enhanced dissociation and thus an improved electrochemical performance.

Based on the understanding gained of the nature of Mg(BH$_4$)$_2$ in solution, we hypothesized that electrochemical performance would be enhanced when the association within the ion pair is weakened. To achieve this, an additive that has an acidic cation with the following characteristics is desirable: 
1) reductive stability comparable to Mg(BH$_4$)$_2$, 2) nonreactive, 3) halide free, and 4) soluble in DME. Hence, LiBH$_4$ was selected as it fulfills all of the above criteria. Mg deposition and stripping was studied in DME using different molar ratios of LiBH$_4$ to Mg(BH$_4$)$_2$; Figure 3a shows the cyclic voltammogram obtained for 3.3:1 molar LiBH$_4$ to Mg(BH$_4$)$_2$ in DME. The use of LiBH$_4$ resulted in an increase of two orders of magnitude in the current density (i.e. oxidation peak current $J_p = 26$ mA cm$^{-2}$), and in a higher coulombic efficiency of up to 94%. We attribute the deposition/stripping currents solely to Mg because of the absence of Li after galvanostatic deposition (Figure 3b), and also the lack of electrochemical activity in a LiBH$_4$/DME solution (Figure S8a). The ionic character of BH$_4^-$ was enhanced, as evidenced by lower vB–H$_4$ and higher vB–H$_n$ bands in the IR spectrum (Figure 3c), thus implying that LiBH$_4$ has a role in increasing Mg(BH$_4$)$_2$ dissociation (the B–H bands for LiBH$_4$/DME occur at lower values, Figure S9). The coulombic efficiency was proportional to the molar ratios of LiBH$_4$/Mg(BH$_4$)$_2$ (Figure S10). A rechargeable Mg battery with a Chevrel phase Mo$_6$S$_8$ cathode, an Mg metal anode, and this optimized electrolyte (Figure 4) demonstrated reversible cycling capabilities at a 128.8 mAg$^{-1}$ rate (capacity retention and cathode magnesiation are shown in Figure S11 and Figure S12). We are currently investigating the sources of the overcharge and capacity fade.

In summary, unprecedented reversible Mg deposition and stripping from an inorganic and relatively ionic salt was obtained in THF and DME. Higher current density and lower overpotentials were achieved in DME compared to those in THF. Substantial enhancement in the coulombic efficiency and the current density was accomplished by the addition of LiBH$_4$. Battery performance was demonstrated using a Chevrel phase cathode. Although the oxidative stability (1.7 V vs. Mg on platinum) currently limits Mg(BH$_4$)$_2$ utilization with high voltage cathodes, utilization with high voltage cathodes, reversibility in the absence of halides and THF makes this salt extremely unique and these findings very important for designing a whole new class of Mg(BH$_4$)$_2$ based electrolytes. Currently, we are investigating improving the oxidative stability of Mg(BH$_4$)$_2$. In addition, the exact nature of the electroactive species in the presence and the absence of the additive is being studied to guide the design of Mg(BH$_4$)$_2$ based electrolytes. This work provides a stepping stone for extending the applications of Mg(BH$_4$)$_2$ and underscores the beauty and versatility of the chemistry of borohydrides.

**Experimental Section**

Magnesium borohydride (Mg(BH$_4$)$_2$, 95\%) lithium borohydride (LiBH$_4$, 90\%), anhydrous tetrahydrofuran (THF), and dimethoxyethane (DME) were purchased from Sigma–Aldrich. Cyclic voltammetry was conducted in a three-electrode cell with Mg wire/ribbon as reference/counter electrodes. The electrochemical testing was conducted in an argon filled glovebox with O$_2$ and H$_2$O amounts kept...
below 0.1 ppm. Details of the analyses and battery testing conducted are described in the Supporting Information.

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flexible devices with CP coatings can address both mechanical and electrical needs for producing robust MEA devices [12].

Conclusion and Future Outlook
MEAs are used to probe electrically active cells in vitro and in vivo towards a better understanding of neuronal network function. The performance of MEA devices can be improved by modifying planar electrodes with porous, high-surface area materials to transduce physiological signals into a flow of electrons [1]. CPs have shown promise as coatings in MEA devices as they can reduce impedance and improve the long-term stability of the electrodes. Different dopants can be incorporated to tailor CP properties to specific applications. This versatility makes conducting polymers attractive materials to operate at a neuronal interface.

The use of CP materials at the neuronal interface shows promise and will continue to expand. Future work for CP coatings should focus on prolonging electrode life in vivo, which remains a limiting factor for implantable neural microelectrodes. Strategies to prolong life may revolve around further improvements in the flexibility of the entire MEA system and matching mechanical properties across the interface. Ongoing and indepth investigations are required to identify which material properties influence cellular interactions and how these can be optimised. To make progress towards an “ideal” electrode, a multidisciplinary collaborative effort between electrochemistry, physiology and engineering is essential, in order to fulfil the potential of MEAs both as investigational tools and as a platform for treatments.

References

Forum
Transcriptional Engineering of Microalgae: Prospects for High-Value Chemicals
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Microalgae are diverse microorganisms that are of interest as novel sources of metabolites for various industrial, nutritional, and pharmaceutical applications. Recent studies have demonstrated transcriptional engineering of some metabolic pathways. We propose here that transcriptional engineering could be a viable means to manipulate the biosynthesis of specific high-value metabolic products.

Biotechnological Applications of Microalgae
Microalgae are highly diverse organisms that are found in many environments and are able to generate a wide variety of potentially useful chemicals and metabolites [1]. In addition, microalgae are attractive ‘platform strains’ for the production of foreign compounds such as vaccines. While microalgal lipids, specifically triacylglycerols (TAGs), and carbohydrates have been of interest for biofuel production, lipids and also proteins are of nutritional interest. Some species can produce high-value long-chain polyunsaturated fatty acids (PUFAs), such as the ω-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Furthermore, many microalgae are rich in bioactive compounds and high-value pigments including chlorophylls, carotenoids, and terpenes [1]. Photosynthetic microalgae are an attractive, potentially low-cost, and sustainable alternative to microbial cell factories such as bacteria or fungi, owing to their ability to utilize CO₂ and solar energy, and thus generate products without needing organic carbon inputs.

To harness the biotechnological potential of microalgae, improved understanding of metabolic processes and the genetic controls underpinning microalgal metabolism is needed. Conventional genetic engineering strategies to enhance specific metabolites rely on modifying individual genes that encode components of a metabolic pathway, but have had mixed success. In some cases the accumulation or composition of the targeted metabolite can remain largely unchanged. An alternative strategy

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is transcriptional engineering (TE), which aims to modify multiple components of a metabolic pathway simultaneously, such as by engineering regulators including transcription factors (TFs). Higher-plant studies demonstrate the potential of TE for metabolic manipulation, such as to enhance the content of nutritional pigments. For example, specific health-promoting anthocyanins were successfully accumulated in fruits by overexpressing two selected TFs that induced >10 enzymes [2]. Likewise, multiple TFs have been identified as regulators of plant secondary metabolites, including isoprenoid metabolism [3]. While TE has yet to be used to manipulate high-value products in microalgae, research is beginning to show the potential of this approach.

**TF Engineering: Current Status in Microalgae**

TFs regulate the expression of specific target genes by binding to specific DNA motifs within cis elements of the target gene and by interacting with the RNA polymerase to activate or repress transcription. Through knowledge of TFs and their gene targets, the transcriptional control process can be manipulated to alter gene expression profiles, such as to modify components of a metabolic pathway. The molecular tools for microalgal TE are still being developed, but several strategies for TE can be considered, either by modifying a TF, such as by overexpression or mutation, or by modifying TF binding domains within target gene promoters.

Recent studies have begun to use omic approaches to identify TFs in microalgal species, with a focus on lipid metabolism. For example, TFs have been identified as regulators of TAG biosynthesis by acting on components of fatty acid and glycerolipid synthesis, and lipid degradation/remobilization [4.5] (Figure 1A). One such TF is PSR1 from *Chlamydomonas reinhardtii*, which is a key component in controlling nutrient starvation-induced TAG biosynthesis [5,6]. Overexpression of PSR1 increases TAG accumulation without inhibiting growth [5], and PSR1 overexpression can also increase starch biosynthesis [6]. PSR1 regulates carbon storage metabolism by controlling specific lipid and starch metabolism genes, and therefore seems to function as a global regulator of microalgal carbon storage [6]. Thus PSR1 is a potential TE tool to mediate broad carbon metabolism modification. TAG can also be increased in microalgae by heterologous expression of a plant TF (GmDOF4) known to regulate soybean lipid content. In *Chlorella ellipsoidea*, GmDOF4 seems to modulate fatty acid synthesis and alters the fatty acid composition of TAG [7].

An intriguing alternative approach to increase nutrient starvation-induced TAG biosynthesis without compromising cell growth is by controlling cell quiescence. CHT7 is a *Chlamydomonas* TF that acts as a regulatory switch between quiescence (during starvation) and proliferation (during nutrient repletion) such that mutating CHT7 promotes starvation-induced TAG accumulation without limiting biomass [8]. The ability to regulate proliferation in cell culture could have benefits for quality control of many metabolic products.

Increased TAG accumulation is beneficial not only for biofuel applications but also for other industrial and nutritional uses of algal lipids. For species that produce ω-3 PUFAs such as DHA and EPA, increased accumulation of TAGs containing these PUFAs will be beneficial. However, enhancing the proportion of long-chain, polyunsaturated fatty acids is an engineering challenge. Plant transcriptional complexes exist that positively regulate seed ω-3 PUFA content by targeting a fatty acid desaturase [9], indicating the TE potential of microalgal PUFA manipulation.

**Future Pathways for Transcriptional Engineering of Value Products**

Previous microalgal TE studies have aimed to modify carbon metabolism with biofuel applications in mind, but there is broader potential for TE to enhance production of high-value compounds for nutraceutical and pharmaceutical uses. Many isoprenoids such as terpenes, phytol, sterols and carotenoids are synthesized in microalgae [1], and there is much interest in these chemicals as novel fuels, fragrances, or flavorings, while some have bioactive properties, with nutritional and health benefits (Figure 1B).

Taking carotenoid metabolism as an example, it is perhaps surprising that there are few successful examples of carotenoid engineering in microalgae to date. Carotenoids are tetraterpenes derived from phytoene, and include β-carotene, a vitamin supplement with demonstrated health benefits, and astaxanthin, an important pigment and anti-oxidant [1]. A few studies have attempted to enhance carotenoids in microalgae, for example by overexpressing one or more carotenoid biosynthesis (CRT) genes such as phytoene synthase or phytoene desaturase (Figure 2A), either from algae or foreign sources, with mixed and inconsistent success [10]. These mixed results are probably because several rate-limiting steps remain in the pathway. These secondary metabolites are products of complex multi-enzyme pathways that therefore require the upregulation of multiple steps to ensure sufficient carbon flux and metabolite accumulation, as seen with tomato anthocyanin accumulation [2]. Conventional approaches to overcome pathway bottlenecks include the introduction of multiple transgenes to overexpress multiple genes simultaneously, but this can be technically challenging. Can TE therefore provide a solution?

Of the microalgal CRT genes identified, many are transcriptionally regulated by environmental stresses. Although carotenoid TFs are yet to be experimentally confirmed, several TFs in *Chlamydomonas* correlate positively with CRTs, including zeaxanthin epoxidase and carotenoid isomerase [4]. The preferred TE strategy...
would be to identify and manipulate only one or two central regulators of a pathway (Figure 2B). Evaluation of Dunaliella bardawil CRTs identified conserved cis elements in many genes, suggesting the presence of central TFs that could be targets for TE [11]. To realize the potential of TE, more TFs must be identified and characterized, including mapping target
Figure 2. Proposed Strategies for Transcriptional Engineering to Increase Carotenoid Metabolism. (A,B) An overview of the carotenoid metabolism pathway from geranylgeranyl pyrophosphate (GGPP) and key enzymes: phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), lycopene-ζ-cyclase (LCY-E), lycopene β-cyclase (LCY-B), cytochrome P450 ζ-hydroxylase (CYP97C3), cytochrome P450 ξ-hydroxylase (CYP97A5), carotene ξ-hydroxylase (CHY-B), β-carotene oxygenase (BKT), zeaxanthin epoxidase (ZEP), violaxanthin de-epoxidase (VDE). (A) Conventional genetic engineering strategies have attempted to increase carotenoids by single-gene overexpression of PSY, PDS, or BKT. (B) A transcriptional engineering (TE) approach may enable activation of multiple components of the pathway, such as by the identification and overexpression of a central regulatory carotenoid transcription factor (TF), or by promoter mutation of cis elements to allow gene activation by a specific TF. DBD, DNA-binding domain; SSD, signal-sensing domain; TAD, transactivation domain. (C) For a successful TE strategy in microalgae, TFs and their target genes must be identified, for example via transcriptomic data analysis to determine regulatory networks and generate correlations between TFs and target genes.

genes and determining cis elements (Figure 2C). The feasibility of synthetic promoters for driving nuclear gene expression has been demonstrated [12]. If central regulators cannot be found, an alternative approach could be to engineer synthetic cis elements of target CRT genes within a pathway, for instance by using genome-editing tools such as CRISPR/Cas9 [13] to drive gene induction (Figure 2).

For TE to be a viable alternative methodology to conventional approaches, such as nuclear or plastid genome expression of single or multiple endogenous or foreign transgenes, a better understanding of endogenous TF networks is needed. By contrast, foreign TFs such as from plants or other organisms could be expressed in microalgae, and are often functional [7]. Furthermore, in silico design could be used to generate synthetic TFs and binding sites [13]. Finally, alternative routes for post-transcriptional engineering may be viable in microalgae, such as the use of riboswitches. While much research and development will be necessary to further understand and validate the uses of these TE tools, particularly for secondary metabolism, TE is a promising
methodology to be included in the microalgae biotechnology toolkit.

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References
9. Mendes, A. et al. (2013) bZIP67 regulates the omega-3 fatty acid content of Arabidopsis seed oil by activating FATTY ACID DESATURASES. Plant Cell 25, 3104–3116
Recent breakthroughs have reinvigorated the century-old research domain of artificial photosynthesis. Here, we highlight CO₂-reducing and O₂-liberating integrated photobioelectrochemical systems that contain novel enzymatic cathodes and photoanodes. These devices, which are completely self-driven by solar energy with unprecedented efficiency and stability, have important implications for biotechnological research communities.

Artificial Photosynthetic Systems

The change in atmospheric CO₂ concentration from 280 ppm in 1750 to 400 ppm in 2015 can be attributed to anthropogenic factors, including massive deforestation, the Industrial Revolution, and population growth (www.noaa.gov/news/record-annual-increase-of-carbon-dioxide-observed-at-mauna-loa-for-2015). This steep increase in CO₂ concentration is resulting in global warming, rise in pollution and is posing a serious threat to the existence of life on Earth [1]. Thus, there is an essential need to reduce atmospheric CO₂, ideally by transforming it into carbon-based fuels with the help of renewable, clean solar energy. Despite extensive research over the past century on CO₂ reduction via chemical, electrochemical, and bioelectrochemical routes, with a specific focus upon materials and catalysis [2], only a few prototypes have been reported (Table 1).

Artificial photosynthetic systems are light-driven devices that not only digest CO₂ to produce important fuels, but also liberate O₂ to the atmosphere if connected to an oxygen evolution reaction (OER) electrode. However, these systems suffer from instability, low efficiency, and high cost, which are important bottlenecks for their further commercialization [5]. Interestingly, recent breakthroughs offer prospects for overcoming these drawbacks.

Cu/ZnO/Al₂O₃ metal-based catalysts are popular choices for the industrial production of methanol from CO, CO₂, and H₂ at high pressure. Recently, the intermetallic compound catalyst Ni₅Ga₃ was reported to catalyze the same reaction but at ambient temperature and pressure, and with lower production of catalyst-poisoning CO [6]. However, these catalysts comprise the nonabundant material gallium. Therefore, a new focus is on developing renewable biological catalysts or catalysts based on metal-free materials. Bioelectrochemical systems (BESs) are ambient-pressure CO₂ reduction devices that use microbes and enzymes as electrocatalysts. Enzymes can drive energy-expensive processes, such as CO₂ reduction, with high yield and product specificity, and, thus, have recently been studied extensively.

Biocatalytic Systems

Microbial Systems

CO₂-sequestering microbial systems provide physiochemical environments that are naturally well suited both for their enzymes to perform CO₂ conversion and to control enzymatic activity. A recent study demonstrated a microbial electrochemical system [7] that exhibited an energy efficiency of ~10%, which compares favorably to the efficiencies of naturally photosynthetic plants (0.2–2%), microalgae (1–1.5%), and cyanobacteria (0.2–0.3%) [8]. This system used a Co-P alloy as a biocompatible cathode to efficiently split water, resulting in the vigorous evolution of H₂. This H₂ was then used to grow an autotrophic microorganism (the bacterium Ralstonia eutropha), which can fix CO₂ selectively into biomass or alcohols and with high efficiency. A self-healing Co-P alloy (leaching of Co⁺ from the cathode and its deposition on the anode) was exploited as an oxygen evolution anode. Interestingly, a Co-Pi catalyst not only reduced the kinetic overpotential for OER, but also provided stability to the anode against a range of operating potentials. This system was demonstrated to be scalable and was not limited by the purity of CO₂ because R. eutropha readily sequestered atmospheric CO₂, which exists at low partial pressure: the
bacterium sequestered 180 g of CO$_2$ per kWh of applied electricity with a CO$_2$ reduction efficiency of approximately 50%. In addition, *R. eutropha* does not operate for 12 h during the ‘night shift’, making it adaptable to intermittent solar power. The authors of this study also suggested that this BES had a solar-to-chemical efficiency of 9.7% for biomass, 7.6% for bioplastic, and 7.1% for alcohols if coupled with a solar photovoltaic system efficiency of 18%. However, microbial systems usually suffer from various drawbacks, such as low stability when exposed to aerobic environments, time-consuming bacterial growth, expensive product recovery, mixed products as outputs, and lower efficiency in comparison to enzymatic systems.

**Enzymatic Systems**

Recently, Srikant and colleagues [9] proposed a BES that efficiently digests CO$_2$ to produce formic acid with a maximum current efficiency of 12.74% at −0.8 V among three different potentials tested (5.36% at −0.4 V and 9.84% at −1.0 V). This BES used formate dehydrogenase (FDH) as a biocatalyst that preferentially produces formic acid, unlike metal-based catalysts. FDH was immobilized on a graphite-based electrode, but the production of formic acid was limited to a maximum of 40 min. The authors of this study concluded that this short operation time might be due to the denaturation of enzymes as an effect of inefficient immobilization or the migration or nonrecycling of the proton donor NADH. The authors also integrated expensive and nonabundant platinum electrodes to drive the evolution and production of O$_2$ and H$^+$, respectively. Thus, two important setbacks need to be overcome for efficient catalytic behavior: the poor longevity of the bioelectrode and the difficulty in immobilizing enzymes.

Interestingly, a recent study described a self-biased BES, independent of external fossil fuel energy, that reduced CO$_2$ to important fuels and chemicals (Figure 1) [10]. The authors of this study fabricated a novel biocathode through electropolymerization, where enzymes and cofactors were deeply embedded in the matrix of polydopamine (PDA) on the surface of the cathode in the form of thin films. This multifunctional PDA not only serves as a favourable matrix for immobilizing enzymes and its cofactors, but also provides them with a suitable physicochemical environment. This design led to unprecedented stable enzymatic catalysis for approximately 2 weeks, making it the longest-lived CO$_2$-reducing biocathode ever reported. The nano-scale thickness of PDA-EC films ensures the easy accessibility of the enzyme to the reactant and effortless interfacial diffusibility for the products. The biocathode was successfully integrated with a visible light-driven anode photocatalyst, BiVO$_4$, to generate electron-hole pairs through irradiation and was coupled with the oxygen evolution cocatalyst Co-Pi for effective water splitting.

This complete prototype is the first reported self-biased artificial photosynthetic system driven by visible light. The authors of the study described the system as an integrated photobioelectrochemical system (IPBES). This IPBES steadily produced formate from CO$_2$, with an ideal Faradaic efficiency of 99.18 ± 6.77%, undergoing little degradation for at least 1 day under standard solar-equivalent illumination. The Faradaic efficiency measures product specificity, quantifying the total charge used in a particular reaction as the percentage of input charge by an external circuit. The solar-to-fuel conversion efficiency was 0.042%, three times lower than that of other abiotic formate-producing systems [11]. Additionally, this prototype has tremendous potential for improvement by engineering the band gap, manipulating the transportation pathway of electrons, and improving the photoanode by developing a more efficient photocatalyst. The efficiency could also be improved by increasing the surface area of the electrode, thereby enhancing the coverage of high-quality immobilized enzymes without altering the thickness of the PDA film. This immobilization technique is applicable to microbes or organelles in addition to enzymes, making these types of BES more durable and productive [12]. Additionally, various other CO$_2$-reduced solar
chemicals and fuels, such as methanol and methane, can be synthesized using similar methods.

**Concluding Remarks**

Researchers have been trying to develop artificial photosynthesis for more than a century [13], but the practical feasibility of the system is still questionable, illustrating the difficulty in mimicking the most important photobiological process on Earth. Earlier studies exploited power sources based on fossil fuels to drive the required processes, whereas the IPBES described above is the first stable enzymatic system to be completely driven by solar energy, signaling a paradigm shift in this field. Other lucrative avenues may further advance the field; for example, tandem IPBESs, where electrocatalysis at both electrodes would be light driven, would improve the efficiency of the systems. A light-driven biocatalyst driven by solar energy, signaling a paradigm shift in the field;

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**References**

Post-Polymerization Modification of Branched Polyglycidol with \(N\)-Hydroxy Phthalimide to Give Ratio-Controlled Amino-Oxy Functionalized Species

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ABSTRACT: Ratio-controlled amino-oxy functionalized, branched polyglycidols are prepared by a post-polymerization modification using and optimizing the Mitsunobu reaction for this purpose. The hydroxyl side-groups are functionalized with \(N\)-hydroxy phthalimide and the hydrazinolysis of this group furnishes a new class of branched polyglycidols with pendant amino-oxy groups. Reproducible functionalization degrees of 17, 33, 43, and 63% of the hydroxyl groups are obtained via the presented developed methodology. MTT assays demonstrate the biocompatibility of amino-oxy functionalized materials. With this, the prepared structural motifs are valuable precursors for the synthesis of biomaterials, bioconjugates and hydrogels in which orthogonal strategies are desired. © 2016 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. 2016, 54, 2820–2825

KEYWORDS: amino-oxy polyglycidol; materials science; polyglycerol; polyglycidol; polymers; post-polymerization functionalization; ring-opening polymerization

INTRODUCTION Advanced polymeric materials greatly depend on the availability of functionalized building blocks to facilitate novel properties and applications. Branched polyglycidols have the make-up to be an ideal precursor for post-polymerization modification strategies, providing unprecedented diversity and value for the synthesis of desired hydrophilic and functionalized building blocks. Developed as an alternative to highly branched polyglycidols as previously reported, the featured polyglycidols with lower branching allow for an increased number of hydroxyl groups available, while still possessing the advantages associated with its branched structure. The branching and shorter molecular weight can provide a reduced toxicity and immunogenicity in contrast to linear, high molecular weight PEG units and also guarantees a facile modification and implementation into more complex systems. Furthermore, these polyglycidols display hydroxyl groups in close proximity and different connectivity, offering a more selected placement of functional groups throughout the structure than possible with linear counterparts. In this work, we will report on the first post-polymerization synthesis of amino-oxy-functionalized branched polyglycidols using the Mitsunobu reaction to install the \(N\)-hydroxyphthalimide in controlled ratios, followed by hydrazinolysis (Scheme 1). An optimization of often times difficult Mitsunobu reactions and thorough characterization methods overcomin some of the reported challenges in the identification of functionalized hyperbranched structures is presented. This structural motif can serve as a branched, PEG-like precursor for the synthesis of biomaterials, bioconjugates and hydrogels where rapid crosslinking without requiring outer stimulus is desired.

Amino-oxy groups are of interested due to their enhanced nucleophilicity compared to the amino group and reacts chemoselectively with aldehydes and ketones to form oximes under mild conditions in the presence of a catalyst or even at physiological pH. The high chemospecificity makes this functionality well suited as bioorthogonal unit for conjugation to proteins and peptides to polymers, synthesis of drug conjugates and various conjugates of carbohydrates and the detection of carbonyl compounds in environmental water samples. To this extend, amino-oxy functional groups have also been utilized in the synthesis of oxime hydrogels for drug delivery and tissue engineering applications as Maynard and Sumerlin among others have reported and underlines the importance of this reactive group. The amino-oxy functionality can be synthesized by utilizing a Mitsunobu reaction to incorporate the \(N\)-hydroxyphthalimide moiety followed by cleavage with hydrazine to furnish the desired amino-oxy functionality. Other methods such as the alkylation of ethyl \(N\)-hydroxyacetimidate with alkyl methanesulfonates followed by deprotection of the

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amino-oxy group,22 base catalysed alkylation of alkyl halides with N-hydroxysphthalimide followed by subsequent hydrazinolysis,18 and coupling of Boc protected amino-oxy acetic acid followed by deprotection of the Boc group11,23,24 have also been utilized to install the amino-oxy group. However, none of these methods have been applied to the functionalization of hydrophilic, branched structures such as the branched polyglycidol reported in this contribution.

EXPERIMENTAL

Materials
Glycidol (Aldrich, 96%) and N,N-dimethylformamide (Aldrich, 99.8%) was freshly distilled from calcium hydride prior to use. 3-methyl-1-butanol (Aldrich, anhydrous, 99%), Tin (II) trifluoromethanesulfonate (Strem Chemicals, 99%), methanol (Acros, anhydrous, 99.8%), dichloromethane (Aldrich, anhydrous, 99.8%), triphenylphosphine (Aldrich, anhydrous, 99%), N-hydroxyphthalimide (Aldrich, 99%), N,N'-dimethylformamide (Aldrich, 99%), triphenylphosphine oxide (Aldrich, 99%), 2,4,6-triisopropylbenzophenol (Aldrich, 99%), diisopropyl azodicarboxylate (Aldrich, 98%), and hydrazine (Aldrich, anhydrous, 98%) were used as received. Dialysis membranes (Spectrum Laboratories, 4000 Da) PTFE syringe filters were obtained from Thermo Scientific (0.45 μm, Teflon plus glass). All reactions were carried out under argon unless otherwise noted.

Instrumentation
Nuclear magnetic resonance (NMR) were acquired on a Bruker DRX-500 (500 MHz), Bruker AV-400 (400 MHz), or Bruker AV II-600 (600 MHz) instrument (equipped with a 5 mm Z-gradient TCI cryo-probe). Chemical shifts are measured relative to residual solvent peaks as an internal standard set to δ 2.50 and δ 39.52 ([CD3]3SO). Quantitative 13C NMR was performed by using inverse-gated proton decoupled 13C NMR with a delay time of 10 s. A sample concentration of approximately 150 mg/mL was used for analysis. FT-IR spectra were recorded on a Thermo Nicolet IR 100 spectrophotometer and are reported in wavenumbers (cm⁻¹). Compounds were analyzed as neat films on a NaCl plate (transmission). Size exclusion chromatography (SEC) analysis was carried out using Waters Ultrahydrogel™ columns (7.8 × 300 mm, Ultrahydrogel™ 120, Ultrahydrogel™ DP 120Å, Ultrahydrogel™ 250). HPLC grade water (containing 1 mg/mL LiBr) was used as the eluent at a flow rate of 1.0 mL/min at 45 °C. Molecular weights (Mn and Mw) and molecular weight distribution were calculated from poly(ethylene glycol) standards provided by Varian. Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-ToF MS) was carried out on a Voyager DE-STR mass spectrometer equipped with a nitrogen gas laser (337 nm). α-Cyanohydroxycinnamic acid was used as the matrix and NaI was used as the cationization agent. The samples (5 mg/mL in methanol), matrix (20 mg/mL in methanol), and cationization agent (20 mg/mL) were mixed in a 1:1:0.1 ratio respectively and spotted unto a stainless steel sample plate. The accelerating voltage was set to 23,000 and measurements took place in reflector mode. Poly(ethylene glycol) was used for external calibration immediately before the measurement. The data was processed using Data Explorer. All spectra were processed (baseline correction, noise filter and Gaussian smooth) before importing mass list into an excel spreadsheet for data analysis.

Synthesis of Branched Polyglycidol Homopolymer (PGly-5)
To a 25 mL jacketed three-neck-round bottom flask fitted with an argon balloon was added isoamyl alcohol (110 μL, 1.04 mmol) and tin (II) trifluoromethanesulfonate (5.60 mg, 5.60 mg, 1.35 × 10⁻² mmol). The mixture was stirred for 10 min using a mechanical stirrer to form a homogeneous suspension. The jacketed flask was connected to a chiller and cooled to 0 °C. Glycidol (1.8 mL, 27 mmol) was added via syringe pump (0.5 mL/h) and the mixture was stirred for 24 h at which time stirring was no longer possible due to the viscosity of the reaction mixture. Methanol (5 mL) was added to the reaction to dissolve the polymer and sodium bicarbonate solution (5 mL of a 1 mg/mL) was added. The reaction was filtered (0.2 μm PTFE) and precipitated twice in acetone to isolate the polymer. Further purification by dialysis with Spectra/Por dialysis membrane (MWCO = 1000 Da) in water was performed if needed to furnish the desired polymer PGly-5 in 71% yield. IR (film) 3390, 1263, 1115 cm⁻¹.
Synthesis of N-Oxypthalimide Polyglycidol Derivative (P(GlyOP-o-co-G)) (1a)

To a 100 mL round bottom flask fitted with an argon balloon and containing a solution of polyglycidol (PGly-5, 1.7 g, 1.17 mmol) in DMF (20 mL) was added N-hydroxypthalimide (0.954 g, 5.85 mmol) followed by triphenylphosphine (1.53 g, 5.85 mmol) at RT. Disisopropyl azodicarboxylate (1.15 mL, 5.85 mmol) was then added dropwise at 0 °C. The resulting mixture was allowed to warm up to RT and stirred for 24 h. The reaction was concentrated under reduced pressure and precipitated twice in ether:ethyl acetate (1:1) to obtain 1.9 g of the desired polymer 1a as an off-white amorphous solid. IR (film) 3455, 3061, 2919, 1789, 1730, 1373, 1127, 731 cm⁻¹.

Synthesis of Amino-oxy Polyglycidol Derivative (P(G6O-o-co-G)) (2a)

To a 100 mL round bottom flask equipped with a stir bar and an argon ballon was added N-oxyphthalimide polyglycidol 1a (1.0 g). Methanol (25 mL) followed by an excess of anhydrous hydrazine (4.5 mL, 140 mmol) was added and the reaction was allowed to stir for 12 h at RT. The reaction mixture was filtered through 0.45 μm PTFE filter to remove the white solid byproduct and allowed to stir for an additional 6 h at RT. All volatile components were removed under rotary evaporation. The residue was suspended in methanol and filtered a second time. The filtrate was transferred to a dialysis tubing (MWCO = 1000 Da), and was dialyzed against water for 36 h. After dialysis, the product was concentrated to furnish 690 mg of the desired polymer. IR (film) 3407, 2873, 1373, 1113 cm⁻¹.

RESULTS AND DISCUSSION

Branched polyglycidol was synthesized by cationic ring opening polymerization of glycidol using isoamyl alcohol as the initiator and tin triflate as the catalyst (Table S1, Supporting Information). By varying the ratio of initiator to monomer, we intended to elucidate the molecular weight control when targeting for a higher and lower degree of polymerizations (DP) reaching from 80 to 26. We found that the experimental DP values were generally approximately double of the theoretical values accompanying a lower degree of branching (DB) when targeting a lower DP. For example, a theoretical DP of 80 gave a DP of 160 with a DB of 0.53 in contrast to a DB of 0.45 when a DP of 26 was anticipated and a value of 52 obtained (Table S1, Supporting Information). This series of experiments represent the first example of a systematic correlation of a monomer/initiator feed to the resulting molecular weights in cationic polymerizations of polyglycidol. Molecular weights varying from 1000 Da to 4000 Da, with fairly narrow molecular weight distribution (Mw/Mn = 1.3-1.4) were detected via SEC. For the post-polymerization functionalization experiments we chose the polyglycidol sample (PGly-5) with the lowest DB and DP. It is to note, that 19F NMR analysis showed the absence of any trifluoromethanesulfonate in the final product, which would indicate a successful removal of the catalyst (Fig. S13 Supporting Information). Furthermore, a correlation of the SEC data to the MALDI-ToF MS analysis could be drawn (Table S1, Supporting Information) for all samples. However, polyglycidols with a lower DB revealed more homogenous spectra in comparison to spectra associated with a higher DP, which is also one of the reasons to select polymers generated by targeting a lower DP with molecular weights of around 2000 Da. The degrees of branching as well as the composition of each structural unit in the polymer was determined by the use of inverse-gated proton decoupled 13C NMR and are summarized in Table S2, Supporting Information.

Polyglycidols obtained by polymerization of 26/1 (Gly/IAOH), PGly-5, were reacted with substoichiometric amounts of N-hydroxypthalimide, triphenylphosphine, and disisopropylazodicarboxylate (DIAD) with targeted ratios of reagents to hydroxyl groups being 0.25, 0.40, 0.55, and 0.70 to achieve a degree of functionalization of 17%, 33%, and 43% and 63%. The desired polymers were isolated by multiple precipitation in a mixture of ethyl acetate and ethyl ether prior to analysis by SEC, MALDI-ToF mass spectroscopy, 1H, 13C, and 31P NMR spectroscopy. SEC analysis using DMF as the eluent showed that aggregation was observed when SEC analysis was conducted in the absence of LiBr as an additive. The aggregation was due to hydrogen bonding between the hydroxyl and carbonyl groups of different polymer chains. In fact, aggregation could be seen in the SEC analysis of the branched polyglycidol in DMF without LiBr added, due to...
hydrogen bonding between hydroxyl groups. Both $^1$H and $^{13}$C NMR spectroscopy of poly[(glycidol-N-oxyphthalimide)-co-glycidol] ([PGNP]-co-G) exhibit characteristic signals for the phenyl protons attached the N-oxyphthalimide moiety indicating successful incorporation of the desired functionality (Figs. 1 and 3, and Figs. S5–S6, Supporting Information). In the $^1$H NMR, this signal appears as a broad singlet at 7.76 ppm due to significant overlapping of the neighboring peaks. In $^{13}$C NMR, the signal corresponding to the carbon of the carbonyl groups can be seen at 164 ppm and the carbons of the phenyl ring appears at 135, 129, and 124 ppm. There was noticeable shifting of the signals in the polymer backbone region of the $^{13}$C NMR spectrum. Moreover, we could observe significant shifting of the signals corresponding to the Terminal 1 (T1), Terminal 2 (T2) and linear 1,3 structural unit (L1,3) while the linear 1,4 structural unit (L1,4) appears to be unmodified. The resulting decrease in the signal intensity for the primary hydroxyl groups corresponded to an increase in the ratio of NHP to hydroxyl group in the feed. This would indicate that majority of the primary hydroxyl groups (L1,3) participated in the reaction. Due to extensive overlapping, specific assignment of all the peaks was difficult. In $^{31}$P NMR, peaks corresponding to triphenylphosphine oxide were observed as a singlet at 28 ppm. This suggested that trace amounts of triphenylphosphine oxide was present and was removed during purification in the next step. It was also observed that some of the triphenylphosphine (PPh$_3$) had reacted with the polyglycidol as indicated by broad peaks at 31 ppm. This side reaction was reduced by cooling the reaction to 0 °C and adding the DIAD immediately after the addition of PPh$_3$, then allowing the reaction to warm to room temperature and stir over the course of 24 h. The degree of functionalization was calculated by comparing the signal intensity of the phenyl groups on the N-oxyphthalimide moiety to that of the branched polyglycidol backbone consulting both the $^1$H and $^{13}$C NMR. For $^{13}$C NMR an inverse-gated proton decoupled pulse sequence was used with a relaxation delay of 10 s. This allowed for integration of the signal intensities in the $^{13}$C NMR spectrum. For the series of experiments carried out, the degree of functionality was in good agreement with the ratios adjusted in the feed reaching from 0.25, 0.40, 0.55, to 0.70 (Fig. 1). In contrast to the unmodified branched polyglycidol, the solubility of the N-oxyphthalimide derivative varied based on the degree of functionality. Derivatives with ratio of functionalization of [NHP]/[OH] with less than 0.20 had a solubility similar to that of the parent polyglycidol. However, polymers with degrees of functionalization between 0.20 and 0.45 was neither soluble in alcoholic nor chlorinated solvents. For these polymers, a mixed solvent system had to be used to fully dissolve the polymers. Polymers with a degree of functionalization greater than 0.45 were soluble in chlorinated solvents along with tetrahydrofuran and 1,4-dioxane. This variation of solubility supports the successful incorporation of the desired functionality into branched polyglycidol with the ability to tailor the polymer to the desired degree of functionalization. The $N$-oxyphthalimide functionalized polymer was then subjected to hydrazinolysis to yield the poly[(glycidol-N-amino-oxy)-co-glycidol] ([PGAO]-co-G). This reaction was carried out as a suspension in methanol or as a solution in a mix solvent system of methanol and tetrahydrofuran (1:1). This furnished the desired amino-oxy functionalized semibranched polyglycidol in 50–70% yield. The lack of the phenyl protons in the $^1$H NMR as well as the carbon atoms of the phthalimide moiety in the $^{13}$C NMR indicates the reaction was successful (Fig. 3, S9–S10, Supporting Information). It was observed that when a methanol/dichloromethane mixture was used, signals corresponding to a sp$^2$ methylene group were observed in both the $^1$H and $^{13}$C NMR spectrum (Fig. S14, Supporting Information) and was attributed to the reaction of the amino-oxy group with dichloromethane which was circumvented by replacing DCM with tetrahydrofuran. Additionally, supporting a strong hydrogen bonding effect, the amino-oxy polyglycidol would take a long time to dissolve in water and analysis by SEC in water showed significant aggregation (Fig. 2). It has to be pointed out that the aqueous solutions appeared not to be heterogeneous. However, MALDI-ToF MS analysis showed a correlation with the molecular weights obtained from SEC analysis (Fig. 2, S12 Supporting Information). As expected, the molecular weight distribution did not correlate as well due to the observed hydrogen-bonding of the polymer sample during SEC analysis.

As we plan to utilize these materials in biological environments, we studied the cytotoxicity of amino-oxy polyglycidols...
Characterization of the series of \( \text{P}[(\text{GAO})\text{-co-}G] \), with SEC analysis carried out in water (containing 1 mg/mL LiBr) with a flow rate of 1.0 mL/min at 45 °C using poly(ethylene glycol) standards (top). The table (below) summarizes the MALDI-ToF, the quantitative NMR analysis and the polydispersity values for the different degree of functionalization after deprotection. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### Table

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<th>Sample</th>
<th>Degree of Functionalization by (^1H) NMR/( % )</th>
<th>GPC</th>
<th>MALDI-ToF MS</th>
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<tr>
<td>2a</td>
<td>17</td>
<td>2500</td>
<td>1.64</td>
<td>1600</td>
</tr>
<tr>
<td>2b</td>
<td>33</td>
<td>2100</td>
<td>2.01</td>
<td>2000</td>
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<tr>
<td>2c</td>
<td>43</td>
<td>1700</td>
<td>2.52</td>
<td>2500</td>
</tr>
<tr>
<td>2d</td>
<td>63</td>
<td>1900</td>
<td>2.08</td>
<td>2500</td>
</tr>
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**FIGURE 3** \(^1H\) NMR overlay of representative examples of the intermediate \( \text{P}[(\text{GNOP})\text{-co-}G] \) and final product \( \text{P}[(\text{GAO})\text{-co-}G] \). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**FIGURE 4** (A) Cytotoxicity of polymers 2b and 2d \( \text{P}[(\text{GAO})\text{-co-}G] \) against NIH 3T3 mouse fibroblast cells at increasing concentrations after a period of 24 and 96 h. (B) MTT assay of polymer 2b showing increased reduction over time at increasing concentration, which indicates that cells are proliferating. The data is displayed as the average and standard deviation of three independent trials for each test condition. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
with 33% (2b) and 63% (2d) degree of functionalization against mouse fibroblast cells using the MTT assay. The cells were incubated with increasing concentrations of polymer 1, 5, and 10 mg/mL for 24 and 96 h before undertaking the MTT assay. The amino-oxy functionalized branched polyglycidol showed very little toxicity toward fibroblasts, with about 78% cell viability observed for the highly functionalized polymer 2d, even at a high concentration of 10 mg/mL after 96 h of incubation [Fig. 4(A)]. Furthermore, increasing the amount of the amino-oxy functionality on the polymer showed very little effect on toxicity. The data also reveal that the cells are proliferating at all concentrations tested as indicated by the increased reduction of MTT after 96 h [Fig. 4(B)]. These results indicate that the method used to functionalize these polymers result in biocompatible building blocks for the preparation of biomaterials.

CONCLUSIONS

In summary, we have successfully applied and optimized the Mitsunobu reaction toward the synthesis of a new class of complex semibranched polyglycidol derivatives with pendant amino-oxy groups in a post-polymerization approach. This convenient synthetic route is suitable for tailoring the amino-oxy groups in a post-polymerization approach. The amino-oxy functionalized branched polyglycidol showed very little toxicity toward fibroblasts, with about 78% cell viability observed for the highly functionalized polymer 2d, even at a high concentration of 10 mg/mL after 96 h of incubation [Fig. 4(A)]. Furthermore, increasing the amount of the amino-oxy functionality on the polymer showed very little effect on toxicity. The data also reveal that the cells are proliferating at all concentrations tested as indicated by the increased reduction of MTT after 96 h [Fig. 4(B)]. These results indicate that the method used to functionalize these polymers result in biocompatible building blocks for the preparation of biomaterials.

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REFERENCES AND NOTES

Growth and Characterization of Conducting ZnO Thin Films by Atomic Layer Deposition

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ZnO thin films were grown on Si or SiO$_2$/Si substrates, at growth temperatures ranging from 150 to 400 °C, by atomic layer deposition (ALD) using diethylzinc and water. Despite the large band gap of 3.3 eV, the ALD ZnO films show high $n$-type conductivity, i.e. low resistivity in the order of $10^{-3}$ Ω cm. In order to understand the high conductivity of ALD ZnO films, the films were characterized with X-ray diffraction, transmission electron microscopy, X-ray photoelectron spectroscopy, elastic recoil detection, Rutherford backscattering, Photoluminescence, and Raman spectroscopy. In addition, the various analytical data of the ZnO films were compared with those of ZnO single crystal. According to our analytical data, metallic zinc plays an important role for the high conductivity in ALD ZnO films. Therefore when the metallic zinc was additionally oxidized with ozone by a modified ALD sequence, the resistivity of ZnO films could be adjusted in a range of $3.8 \times 10^{-3} \sim 19.0$ Ω cm depending on the exposure time of ozone.

Key Words: ZnO, Conducting, Atomic layer deposition, Thin film

Introduction

Zinc oxide (ZnO) is an $n$-type semiconductor with a wide band gap of 3.3 eV.$^{1,2}$ Recent progress in processing ZnO has opened up numerous applications for varistors, phosphors, sensors, UV light emitters, transparent high power electronics, surface acoustic wave devices, piezoelectric transducers, and optoelectronic devices.$^3$ Not only bulk crystal and thin films of ZnO but nano-structured ZnO such as nanowires and nanoparticles have been also intensively studied for various applications.$^4$

ZnO thin films have been grown by various methods such as spray pyrolysis,$^6$ sol-gel,$^1$ sputtering,$^7$ chemical bath deposition,$^8$ metal-organic chemical vapor deposition,$^9$ and atomic layer deposition (ALD).$^{10,11}$ The ALD method is a special modification of chemical vapor deposition for self-limiting film growth.$^1$ The precursor vapor and reaction gas are alternately pulsed onto a substrate. The reaction chamber is purged with an inert gas between the pulses of precursor vapor and reaction gas. All the process steps are performed at low temperature, usually lower than 400 °C, to avoid thermal decomposition of the chemisorbed precursor molecules.

Assuming the hypothetical situation of a perfect pure single crystal without any defects, ZnO would be an insulator rather than a semiconductor at room temperature.$^2$ However, in the practically-obtained single crystal, the resistivity of ZnO is in the order of $10^5$ Ω cm.$^{12}$ Furthermore, ZnO thin films show a wide range of resistivity variation from $10^{-2}$ to $10^5$ Ω cm, depending on the growth process condition.$^{1,13,14}$ Carcia et al.$^2$ showed that the resistivity of ZnO films is increased from $10^{-2}$ to $10^5$ Ω cm by increasing oxygen partial pressure in sputtering method.$^1$ Kohiki et al.$^5$ also showed that the resistivity (10$^5$ Ω cm) of an as-grown ZnO film was dramatically decreased to $10^{-3}$ Ω cm for a film doped by hydrogen implantation.$^{18}$

Because most ZnO is strongly $n$-type, it has been long assumed that the dominant donor is a native defect, either the oxygen vacancy (V$_O$), or the zinc interstitial (Zn$_i$.$^{20}$ However, the V$_O$ is no longer considered to contribute to the high conductivity because its energy level is too deep in the band gap of ZnO.$^{21,25}$

Recently, Van de Walle reported that the cause of the high conductivity of ZnO is hydrogen which incorporates in high concentrations and behaves as a shallow donor.$^21$ The role of hydrogen was also experimentally examined with sputtered ZnO:H films of which resistivity reached $2 \times 10^{-4}$ Ω cm.$^{23}$

On the other hand ZnO films grown by ALD generally show high $n$-type conductivity with a low resistivity in the order of $10^{-3} \sim 10^{-1}$ Ω cm.$^{10,11}$ However, the high $n$-type conductivity of the ALD ZnO films is not clearly understood yet due to the complexity of its transport behavior and lack of characterization for the ALD ZnO films. In this report, ZnO films were grown by ALD using diethylzinc (DEZ) and water as a Zn precursor and an oxidant, respectively. We investigated the growth behavior and electrical properties of ZnO films. The films were characterized to understand the high conductivity of ALD ZnO by various analytical methods such as X-ray diffraction (XRD), transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), elastic recoil detection (ERD), Rutherford backscattering (RBS), Photoluminescence (PL), and Raman spectroscopy. In addition, the various analytical data of the ALD ZnO films were compared with those of a single crystalline ZnO. The effect of oxidant on the conductivity of ZnO films was also investigated by using ozone as an additional oxidant.

Experimental Section

ZnO thin films were deposited on 8-inch bare silicon wafers for Figure 1 or SiO$_2$ (100 nm)/Si substrates for other figures, at deposition temperatures ranging from 150 to 400 °C, by using DEZ and water. The feeding time of water vapor was 2 s, and that of DEZ was varied from 0.1 to 3 s to confirm the self-
limiting growth behavior as shown in Figure 1b. The purging times were 5 seconds under Ar flow of 1600 sccm. DEZ and water were vaporized at room temperature, and delivered without any carrier gas to the reactor. The reactor pressure during the ALD process was around 0.6 Torr.

The film thickness and refractive index were determined by a spectroscopic ellipsometer (J. A. Woollam Co., Inc.) with a Cauchy dispersion model. The preferred orientation and crystal structure were examined by XRD measurements in 0/20 scan mode with Cu Kα radiation and cross sectional high resolution TEM. For a comparison with the ALD ZnO films, an O-face polished ZnO single crystal (MTI Corp., 99.999%, <0001>) was used as a reference for XPS, PL and Raman spectroscopy. XPS measurements were performed on a Quantum 2000 Microprobe PHI spectrometer using a monochromatized Al Kα emission. Binding energies were measured using the C 1s peak (284.8 eV) of the adventitious carbon as an internal standard. Sputtering during the XPS depth profile was performed with 0.5 keV Ar+ ions. The O/Zn ratio and hydrogen content in the films were analyzed by RBS and ERD, respectively. The optical properties of ZnO were characterized at room temperature by PL with a He-Cd laser as a light source using an excitation wavelength of 325 nm, and the laser powers were 6 mW/cm² for the films and 2 mW/cm² for the single crystal, respectively. Raman scattering measurements were performed in 180° backscattering geometry by using a 488 nm laser excitation. The carrier-type, concentration and mobility were measured using a Hall measurement with the Van der Pauw electrode configuration under a magnetic field of 0.5 T at room temperature.

**Results and Discussion**

Figure 1 shows ZnO growth behavior by ALD. The growth rates in Figure 1a and 1b were determined from ZnO films grown on bare Si wafers for 300 and 100 cycles, respectively. The growth rate of ZnO films largely depends on the growth temperature, as shown in Figure 1a, which was also reported by others. In this ALD reactor and process conditions, the growth rate linearly decreases from ~ 2 Å/cycle at 150 °C to 0.53 Å/cycle at 400 °C. From the growth rate uniformity over an 8-inch substrate, it was determined that the proper temperature window of ALD is in a range of 200 ~ 300 °C wherein the standard deviations in thicknesses are lower than 3%. The standard deviation of the film thickness grown at 400 °C is about 16%, possibly due to thermal decomposition of DEZ. Figure 1b shows the variation in growth rate as a function of feeding time for DEZ. The self-limiting growth behavior of ZnO thin films can be confirmed by this experiment. In the low feeding time of DEZ below 0.5 s, the standard deviations of the growth rate are large, but the sufficient supply of DEZ over 2 s gives small standard deviations with a saturated growth rate of ~ 2 Å/cycle at 150 °C.

For characterization of ZnO films, around 50 nm-thick ZnO films were grown on SiO₂ (100 nm)/Si substrates at different temperatures. The refractive index of a bulk ZnO at 589 nm is 2.00 ~ 2.02 but those of ZnO films are smaller than the literature value as shown in Figure 2 (open circles). However, the refractive index increases as the growth temperature approaches the proper ALD region (200 ~ 300 °C).

**Figure 2**. Resistivity (solid circles) and refractive index (open circles) of 50 nm-thick ZnO films on SiO₂ (100 nm)/Si substrates as a function of growth temperature. The error bars denote standard deviations of the values obtained from 9 different positions in an 8-inch substrate.
Conducting ZnO Thin Films by Atomic Layer Deposition

**Figure 3.** Electron mobility (solid circles) and carrier concentration (open circles) as a function of growth temperature.

![Graph showing electron mobility and carrier concentration vs. growth temperature](image)

**Figure 4.** XRD patterns of the ZnO films grown at growth temperatures of 150, 250, 300 and 400 °C.

![XRD patterns of ZnO films](image)

Concentration means that the donor-like defects such as Zn$_{\delta}$ V$_{O}$ and/or hydrogen were highly incorporated in ZnO films. As growth temperature increases over 250 °C, the electron mobility decreases but the carrier concentration increases. The opposite tendency can be explained by the impurity scattering. Electron mobility generally decreases with increasing carrier concentration because the impurity scattering increases in proportional to its carrier concentration. However, the nearly-constant mobility in the growth temperature region of 150 ~ 250 °C reveals that there are other factors which contribute the electron mobility. Those may be grain size and crystallographic orientation of crystallites in the ZnO films as discussed in the following section.

In the XRD patterns of Figure 4, the films grown at higher temperatures than 300 °C show a <002> preferred orientation, of which (002) planes are parallel to the substrate surface. However, those grown at lower temperatures than 250 °C show a random orientation. Figure 5a and 5c show cross sectional TEM images of ZnO films grown at 250 and 400 °C, respectively. It is clearly observed that both films are grown with columnar grains, but the film grown at 400 °C has slightly larger grains than that grown at 250 °C. In scanning electron microscopic and atomic force microscopic images of the film surfaces (see the Supplementary Materials), the grain size is weakly dependent on the growth temperature and the root-mean-square (rms) roughness of the films was 0.5 ~ 1.1 nm. Figure 5b and 5d show high resolution TEM images of ZnO grown at 250 and 400 °C, respectively. The film grown at 400 °C is better crystallized with a more preferred growth orientation than that grown at 250 °C, as also revealed in the digital diffractogram.

Since the wurtzite structure of ZnO is anisotropic in the crystallographic aspect, ZnO shows anisotropic mobility due to piezoelectric scattering. The piezoelectric scattering acts only in the direction of the hexagonal c axis, thereby causing a reduc-

**Figure 5.** Low resolution (a and c) and high resolution (b and d) TEM images of the ZnO films grown at 250 (a and b) and 400 °C (c and d). Insets show digital diffractograms from the corresponding high resolution TEM images.

![TEM images of ZnO films](image)
tion of the carrier mobility by a factor of about 2 compared to the value in the ab plane.\textsuperscript{23} Because the <002> preferred orientation of ZnO films is attenuated at lower growth temperatures, the mobility of the ZnO films with random orientation should decrease as the growth temperature decreases. The nearly-constant mobility at the growth temperature of 150 ~ 250 °C may be originated from a compromise between impurity scattering and piezoelectric scattering.

Figure 6a shows XPS depth profiles of the film (open circles) grown at 250 °C and ZnO single crystal (solid circles). Zn and O in the film are uniformly distributed from the surface to the interface between the film and substrate. The residual carbon content in the film is lower than the XPS detection limit (see the Supplementary Materials for the profiles of other films).

According to the depth profile by XPS, the O/Zn ratio of the film grown at 250 °C is evaluated be 0.77 which is much smaller than one expected from the stoichiometry of ZnO. It seems to be highly oxygen deficient in the film, but it is not true since the matrix effect of ZnO on the sputtering for depth profiling was not considered. Indeed, the O/Zn ratio (0.72) of single crystal is rather smaller than that (0.77) of the film. The low O/Zn ratio by XPS is originated from a preferential sputtering of oxygen to zinc during sputtering.\textsuperscript{23} Therefore, the O/Zn ratio was non-destructively analyzed by RBS as shown in Figure 6b (solid circles). The O/Zn ratio of the ZnO film grown at 250 °C is ~1, but the ratio is rather larger than one in the films grown at higher and lower temperatures. Eventually, the ZnO films by ALD are not oxygen-deficient at least.

In the oxygen rich compositions, it is expected that oxygen interstitials (O\textsubscript{i}) and zinc vacancies (V\textsubscript{Zn}) may play a role of acceptor-like defects resulting in p-type conductivity. However, the mobility and carrier concentration in Figure 3 reveal the n-type nature of the ZnO films grown by ALD, despite their zinc deficiency from the stoichiometry. It was reported through theoretical calculations that ZnO cannot be doped p-type via native defects (O\textsubscript{i} and V\textsubscript{Zn}), since the donor-like defects (Zn\textsubscript{ii} and V\textsubscript{O}) that could compensate p-type doping have low formation enthalpies at both Zn-rich and O-rich conditions.\textsuperscript{24} Recently, Tan, et al. reported the change of conduction type from n to p occurred at an O/Zn ratio of 5 which is highly oxygen-rich.\textsuperscript{26}

Figure 6b (open circles) shows the H contents in the films grown at 150 ~ 400 °C, analyzed by ERD. The H content decreases as the growth temperature increases. This is an opposite tendency to the increasing conductivity and carrier concentration as shown in Figure 2b and 3, respectively. In addition the H content is lower than 0.5 at % in the whole temperature range. This value corresponds to the hydrogen doping level of ~2 × 10\textsuperscript{20}/cm\textsuperscript{3} which is much smaller than the measured carrier concentration in Figure 3. Therefore the incorporated hydrogen may only partially contribute to the n-type conductivity of ZnO films.

Figure 7a shows Zn 2p\textsubscript{3/2} core level spectra of ZnO films and a single crystal. The binding energy of Zn 2p\textsubscript{3/2} peak of the ZnO single crystal is 1021.4 eV, and those of the ZnO films are located in the range of 1021.6 ~ 1021.8 eV, shifting to higher binding energies at higher growth temperatures. However, it is quite difficult to distinguish the oxidation state of Zn in the films with Zn 2p\textsubscript{3/2} peaks, since the 2p\textsubscript{3/2} binding energy range (1020.8 ~ 1022.1 eV) of Zn\textsuperscript{2+} overlaps with that (1021.2 ~ 1022.5 eV) of Zn\textsuperscript{2+} in ZnO.\textsuperscript{31} On the other hand, the Zn LMM

Figure 7. Zn 2p\textsubscript{3/2} (a) and Zn LMM (b) core level spectra of the ZnO films grown at different temperatures and ZnO single crystal. The spectra were obtained after sputtering with Ar\textsuperscript{+} of 0.5 keV for 4 min to remove the contaminant on the surface. (c) Zn2/Zn1 ratio as a function of growth temperature.
Auger spectra are more sensitive to differences in chemical environment than the Zn 2p spectra. The kinetic energy for the LMM peak of Zn\textsuperscript{3+} is ranged in 991.8 ~ 992.5 eV, and the LMM peak of Zn\textsuperscript{2+} is located in the range of 987.7 ~ 988.9 eV.\textsuperscript{31} Figure 7b shows the Zn LMM spectra of ZnO thin films and a single crystal. The lower kinetic energy peaks (Zn\textsuperscript{1+} ~ 988.5 eV) are attributed to the bonding of Zn with oxygen in ZnO, whereas the shoulder peaks (Zn\textsuperscript{2+} ~ 991.7 eV) indicate the presence of metallic zinc originated from Zn\textsubscript{6} and/or V\textsubscript{O}. The metallic Zn to the oxidized Zn peak ratio (Zn\textsubscript{2}/Zn\textsubscript{1}) is plotted as a function of growth temperature in Figure 7c. The Zn\textsubscript{2}/Zn\textsubscript{1} ratios in the ZnO films are much higher than ~ 0.21 in the single crystal as denoted with a dotted line. This reveals that the Zn\textsubscript{6} and/or V\textsubscript{O} are more incorporated in the films than in the single crystal. It should be noted that the Zn\textsubscript{2}/Zn\textsubscript{1} varies with the growth temperature in a similar manner shown by the O/Zn ratio in Figure 6b. Although it is expected that the metallic zinc may be less incorporated in oxygen-richer ZnO films, the Zn\textsubscript{2}/Zn\textsubscript{1} ratio of the films increases with the increasing O/Zn ratio. It may be due to the low formation energies of Zn\textsubscript{6} and/or V\textsubscript{O} even in the oxygen-rich condition.\textsuperscript{29}

Figure 8a shows PL spectra of the ZnO films and the ZnO single crystal. For the single crystal, the strong emission from the band edge is observed at 377 nm due to the free-exciton recombination. For the ZnO films, the near band edge emission peaks are located at 368.0 ~ 380.0 nm shifting to lower wavelengths at higher growth temperatures. The inset in Figure 8a shows the full width at half maximum (FWHM) of the near band emission peaks as a function of the growth temperature. The FWHM of the film grown at 250 °C approaches to that (11.5 nm) of ZnO single crystal which was indicated with a dotted line in the inset.

The ZnO single crystal also shows weak and broad emission in green region. However the green emission is not observed from the ZnO films. It should be noted that the green emission is originated from oxygen vacancies.\textsuperscript{32-34} It is recently reported that the ZnO film grown by ALD nearly does not have oxygen vacancies and does not emit light in the visible region.\textsuperscript{35}

ZnO has a wurtzite structure with C\textsubscript{6h} point group symmetry. There are six Raman active modes: two E\textsubscript{2} vibrations at 101 and 437 cm\textsuperscript{−1}; one transverse A\textsubscript{1} at 381 cm\textsuperscript{−1} and one transverse E\textsubscript{1} at 407 cm\textsuperscript{−1}; one longitudinal A\textsubscript{1} at 574 cm\textsuperscript{−1} and one longitudinal E\textsubscript{1} at 583 cm\textsuperscript{−1}.\textsuperscript{36} If the incident light is exactly normal to the surface of ZnO, only longitudinal A\textsubscript{1} and E\textsubscript{2} modes are observed, and the other modes are forbidden according to the Raman selection rules.\textsuperscript{37} Figure 8b shows Raman spectra of the ZnO films and single crystal. In the ZnO single crystal, strong E\textsubscript{2} and broad longitudinal A\textsubscript{1} modes are located at 438 and 576 cm\textsuperscript{−1}, respectively. The wurtzite phase of ZnO films can be easily characterized particularly with the appearance of the high frequency E\textsubscript{2} mode at 435 cm\textsuperscript{−1}, although the longitudinal A\textsubscript{1} mode peak were not clearly resolved from the Si peak at 520 cm\textsuperscript{−1}. The absence of the E\textsubscript{2} mode at 583 cm\textsuperscript{−1}, which is associated with oxygen vacancy, also supports that the grown ZnO films barely have oxygen vacancies.\textsuperscript{38}

To investigate the effect of oxidant on the conductivity of ALD ZnO films, ALD sequence was modified with ozone which is a stronger oxidant than water. Ozone exposure and purging steps were inserted between purging water and supplying DEZ. Therefore the modified ALD sequence is DEZ (2 s) – purging (5 s) – water (2 s) – purging (5 s) – ozone – purging (5 s) where the exposure time of ozone is varied from 0 to 20 s. ZnO films (50 ~ 60 nm) were deposited at 250 °C on SiO\textsubscript{2} (100 nm)/Si substrates with different feeding times of ozone by the modified sequence. The ozone concentration was 150 g/m\textsuperscript{3} and the oxygen flow for the ozone generation was 800 sccm. Figure 9 shows that the resistivity of the ZnO films by the modified sequence rapidly increases from 3.8 × 10\textsuperscript{3} to 19.0 Ωcm as the ozone feeding time increases from 0 to 5 sec. However the resistivity is saturated at longer exposure times of ozone than 5 s. This reveals that the metallic zinc observed in the Zn LMM spectra may be additionally oxidized by ozone.

**Conclusion**

ZnO films were grown by ALD using DEZ and water as a
Zn precursor and an oxidant, respectively. Resistivity of the films is in the order of $10^{-3} \Omega \text{cm}$ showing a decreasing tendency at higher growth temperatures. In order to understand the high conductivity of ALD ZnO films, the films were characterized by XRD, TEM, XPS, ERD, RBS, PL, and Raman spectroscopy. In addition, the various analytical data of the ZnO films were compared with those of ZnO single crystal. According to our analytical investigation, metallic zinc in the films plays a role for the high conductivity of ALD ZnO. Therefore, the conductivity of ALD ZnO could be decreased by using a modified ALD sequence with additional oxidation step by ozone. This fundamental characterization of ALD ZnO films may facilitate conductivity modulation and $p$-type doping of ZnO for various device applications.

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Supplementary Materials. SEM and AFM images and XPS depth profiles for ZnO films grown at different temperatures are available.

References