Sterically stabilized self-assembling reversibly cross-linked polyelectrolyte complexes with nucleic acids for environmental and medical applications

Martin C. Garnett†, Paolo Ferruti‡, Elisabetta Ranucci‡, Marco A. Suardi‡, Mieke Heyde‡ and Rob Sleat‡

*School of Pharmacy, University Park, University of Nottingham, Nottingham NG7 2RD, U.K., †Department of Organic and Industrial Chemistry, University of Milan, Via Venezian 21, 20133 Milan, Italy, and ‡EnviroGene Limited, Tredomen Innovation & Technology Centre, Tredomen Business Park, Ystrad Mynach, Hengoed CF82 7FQ, Wales, U.K.

Abstract
One of the principal problems facing nucleic acid delivery systems using polyplexes is the instability of the complexes in the presence of proteins and high salt concentrations. We have used a cross-linking polymer to overcome this problem. Pendant thiol moieties have been incorporated into a PAA (polyamidoamine) homopolymer and a PEG (poly(ethylene glycol))–PAA–PEG copolymer reported previously as a self-assembling system. When mixed with DNA, small monodisperse sterically stabilized particles are formed in quantitative yields. Optimization of the formulation resulted in nanoparticles which are stable in seawater. This cross-linked formulation has been successfully tested in both freshwater and estuarine field trials as a water tracer. Future work will develop these particles as a groundwater tracer and also for therapeutic applications of nucleic acid delivery.

Introduction
One of the most difficult problems in medical therapeutics is how to deliver nucleic acids such as genes and siRNA (small interfering RNA) to cells. The problem relates to condensing and protecting a large biodegradable molecule from a hostile environment, while at the same time overcoming barriers of adsorption and penetration of various cell membranes to reach the target. At the end of this process, the nucleic acid needs to be released in an active form [1]. These problems have largely been overcome by viruses, but the use of viruses brings other unwanted consequences. It would be more useful to be able to incorporate the functionality of viruses into a wholly synthetic construct [2]. One path which has been followed to achieve this objective is the use of polymer-based complexes known as polyplexes. These complexes are based on an interaction of the highly negatively charged nucleic acid with a positively charged polymer to form polyelectrolyte complexes. With therapeutically useful sizes of nucleic acid, this is a spontaneous and complete reaction typically resulting in nanometre-sized (20–200 nm diameter) discrete particles.

The theory of polyelectrolyte complexes is well known and has been reported extensively in the literature (e.g. [3]). Most living organisms use a similar process for DNA condensation. A variety of cationic polymers have been used to investigate DNA delivery [4], but few of these polymers are both biodegradable and produce useful well-defined non-aggregating complexes. Preparing polyplexes from a cationic homopolymer and DNA requires an excess of one of the components (typically the cationic polymer), resulting in a particle which is charge-stabilized by the presence of the excess polymer. The presence of the charged surface means that, for some polymers, these particles can exist in a non-aggregated form. However, the presence of the charge means that the particles can readily adsorb to negatively charged substances and surfaces, and that they are destabilized in the presence of salt, resulting in aggregation. To overcome the charge problem, the usual solution is to create a sterically stabilized surface in which a hydrophilic polymer is densely packed on the surface leaving a neutral charge and an entropic barrier to interactions between the particle and other surfaces. The most effective way of achieving this is to chemically attach PEG [poly(ethylene glycol)] to the cationic polymer before formation of complexes [5] or to the surface of pre-formed complexes [6].

Self-assembling PAA (polyamidoamine) polyplexes
In our work, we have extensively investigated the linear PAA s as cationic polymers for DNA delivery [7–11]. This is a family of biodegradable polymers produced from a pair of co-monomers, giving a range of possible charge and structural characteristics [12]. These polymers generally have low cytotoxicity [7,13] and are also easily modified to produce A-B-A
block copolymers with terminal PEG groups. Early work identified a particular PAA produced from MBA (methylene bisacrylamide) and DMEDA (dimethylethylene diamine) which produced well-structured non-aggregating particles and had good biological activity for transfecting DNA [8]. Initial attempts by us to use the PEGylated block copolymer to produce complexes were largely unsuccessful, as these complexes, also known as polynion micelles, did not condense DNA well. Similar findings were made by other groups with other PEG–polycation copolymers. We demonstrated that this was due to an incorrect PEG/cationic polymer ratio, which could be overcome by using appropriate mixtures of homopolymer and PEG copolymer [10]. This methodology resulted in small discrete non-aggregating particles with a neutral surface, indicating that steric stabilization had been achieved (the proposed structure of particles is shown in cartoon form in Figure 1). These nanoparticles formed instantaneously and resulted in an essentially quantitative incorporation of both DNA and cationic polymer.

Despite this success in self-assembly, polyelectrolyte complexes suffer from two major shortcomings. First that they are equilibrium systems like micelles, and secondly that they are unstable (disintegrate) in the presence of high salt concentrations. How far the equilibrium is in favour of particle formation and particle stability are difficult to measure. However, the instability is an acknowledged problem in developing polyplex DNA-delivery systems [14].

Charaterization of non-cross-linked polyplexes
Development of self-assembly cross-linked polyplexes

We wanted to incorporate a disulfide-based cross-linker to allow easy release of the DNA after sampling. Our two-component self-assembling system was an ideal starting point, as disulfide bonds can be formed readily by reacting a reduced thiol group with a thiol group activated by 2-thiopyridine. The introduction of thiol groups was readily accomplished by a new PAA polymer synthesis where some of the amionic component was replaced by a mono-Boc (t-butoxycarbonyl)-protected cystamine [15]. This disulfide-containing polymer can then be readily converted into either the reduced thiol- or thiopyridyl-substituted polymer. To give the two-component system required, PEG–PAA–PEG copolymer (CP) with reduced thiol groups and a short cross-linking homopolymer (XLP) with thiopyridyl activated groups were produced (the polymer structures are presented in Figure 2). Mixing the copolymers with DNA resulted in the spontaneous formation of cross-linked nanoparticles. A cartoon depicting the cross-linked structure is shown in Figure 3. One of the important features of the chemistry used in the production of these cross-linked particles is that the only side product is the release of a small quantity of thiopyridyl group on disulfide bond formation, so no clean-up of product is required. Initial experiments showed that >99% of added oligonucleotide was incorporated into the complexes.

We (M.C.G. and P.F.) were approached by EnviroGene Ltd who wanted to use DNA as a tracer in water courses and also required a protected DNA nanoparticle system. Their requirements for this application were very similar to ours, in that they needed to protect the DNA, condense it into a very large number of small particles, have particles which did not adsorb to materials in the environment and to be readily released to be able to determine the sequence and amount of DNA present in a water sample by qPCR (quantitative PCR). In addition, they needed a biocompatible system which was relatively cheap and easy to manufacture in large quantities. Working together, we have devised and produced an appropriate reversibly cross-linked formulation.

Characterization of cross-linked polyplexes

It was found that the PAA/DNA ratio needed to be close to 1. The XLP/CP ratio needs to vary to accommodate the differing chain lengths of PEG and PAA, but the usable range of ratios is limited because of the need to have a reduced thiol/activated thiol ratio of more than 1 to ensure full cross-linking. To achieve appropriate stability of the nanoparticles, approx. 25% of the PAA monomers needed to have pendant thiol groups. Varying the XLP/CP
Figure 2 | Structures of homopolymer (XLP) and PEG-PAA-PEG copolymer (CP) containing thiol (sulphydryl) blocks prepared for cross-linked nanoparticles

Note that only the right-hand end of the copolymer molecule is shown, the thiol and PEG blocks at the left-hand end of the triblock copolymer have been omitted for clarity.

![Chemical Structures](image)

Figure 3 | Cartoon depicting the cross-linking between the homopolymer (XLP) and copolymer (CP) designed to occur at the surface of the particle through placement of the thiol (sulphydryl) blocks near the terminal PEG moieties

and PAA/DNA ratios resulted in a variety of particle morphologies ranging from toroids to spheres.

We determined the stability of the particles against aggregation by measuring the turbidity of the particles in the presence of sodium sulfate, but it was difficult to find an objective assay for the stability of the particles to disintegration in the present of salt. We eventually found that filtering the particles through a centrifugal ultrafiltration membrane and measuring the release of DNA by qPCR proved to be a good indicator of stability. However, a large-pore-size membrane (>100 kDa) is required to allow the DNA to pass through the membrane. Some release of oligonucleotide from the cross-linked nanoparticles was seen, particularly in the presence of high salt concentrations. In these experiments, we could still detect particles by DLS (dynamic light scattering), but when viewed by transmission electron microscopy, the particles resembled empty cages. Further small modifications to the formulation resulted in particles which now gave an acceptable retention of DNA even in 600 mM salt, equivalent in concentration to seawater.

Manufacture of self-assembling polypeplexes

The self-assembly properties of these particles were important in providing an easy manufacturing process. In small-scale preparation of these nanoparticles using a batch method, factors such as the order of addition of reagents are very important in determining size and quality of nanoparticles. Polymer concentration is also important, with high concentrations resulting in larger particles and aggregated particles. We determined that using a continuous flow mixing method actually resulted in a better particle production with a smaller and more consistent particle size. Because of the relatively low polymer concentration, the two polymer components can be mixed together before assembly without reacting substantially with each other until addition of the DNA, when well-condensed particles are formed. We have currently made nanoparticle batch sizes of up to 210 mg of DNA using this procedure. We believe that this process should be infinitely scalable for larger batch sizes.

Cross-linked polypeplexes as water-borne tracers

Using these cross-linked nanoparticles, field trials have been carried out at both a freshwater and an estuarine site...
to compare the performance of the small DNA tracers with the existing Rhodamine WT dye used in water-tracing applications. Some details of the River Fruin trial are given here. The River Fruin is well channelled, and the trial took place over a 6.1 km stretch of the river. After depositing the dye [2 litres of 20% (w/v) dye] and DNA tracer [1 litre, 10^14 particles (2.5 mg of DNA)] in the river, samples were taken at 10 or 20 min intervals and continued for 22 h. The dye was detected fluorimetrically, and the DNA tracers were detected by qPCR using a 20 μl sample of river water without further treatment. For analysis of DNA, the heating cycle of the PCR process is sufficient to break down the disulfide linkages and release the DNA for enzyme action in the assay. Comparison with the dye showed a similar time to peak for appearance of the DNA tracer at the sampling site at 610 min. The dye became undetectable after the main peak had passed, but the presence of the DNA tracer was detectable well above background for a further 5 h after the main flow peak had passed.

These tests have demonstrated that the new tracers are comparable with the previous dyes in terms of tracing water flow, but have greater sensitivity, and can be used repeatedly and with an infinite number of tracers at the same site, which is not possible with the current dye technology. In addition, DNA does not colour the river visibly as happens which is not possible with the current dye technology. In flow, but have greater sensitivity, and can be used repeatedly and with an infinite number of tracers at the same site, comparable with the previous dyes in terms of tracing water.

The new technology developed for the water-tracing application has many of the characteristics that are necessary to enable its use as a groundwater tracer. Development of the water-tracing technology is in progress with the large amount of dye necessary at present. Further addition, DNA does not colour the river visibly as happens which is not possible with the current dye technology. In and with an infinite number of tracers at the same site, flow, but have greater sensitivity, and can be used repeatedly and with an infinite number of tracers at the same site, comparable with the previous dyes in terms of tracing water.

### References


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