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Author(s)	Aied, Ahmed; Glynn, Barry; Cao, Hongliang; Zheng, Yu; Pandit, Abhay; Wang, Wenxin
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Ahmed Aied, Barry Glynn, Hongliang Cao, Yu Zheng, Hongyun Tai,* Abhay Pandit and Wenxin Wang*

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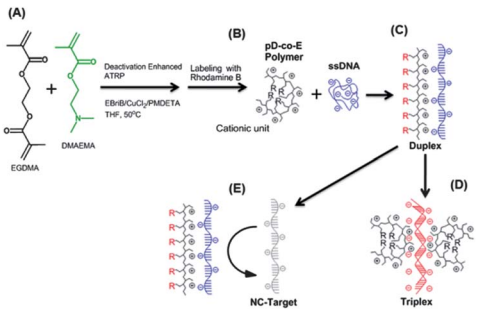

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A fluorescently labeled, hyperbranched polymer synthesized from DE-ATRP for the detection of DNA hybridization†

Ahmed Aied,[a] Barry Glynn,[b] Hongliang Cao,[a] Yu Zheng,[a] Hongyun Tai,*[c] Abhay Pandit[a] and Wenxin Wang*[a]

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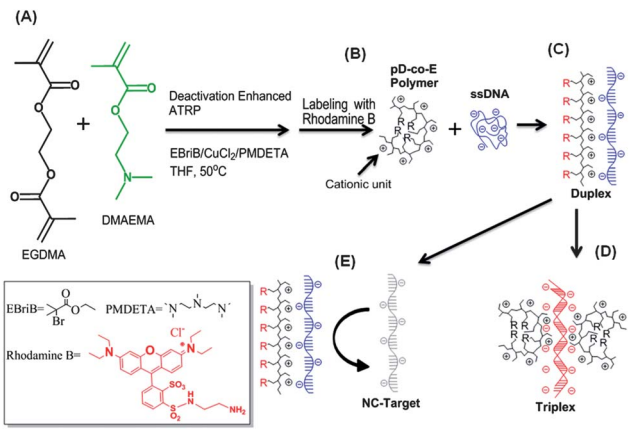
The early detection of oligonucleotide biomarkers of disease, such as microRNAs, has been established as a fundamental factor in cancer diagnosis. As the levels of these small molecules (microRNAs) in blood have recently been found to be significantly affected in cancer patients, they offer a means of early stage detection of cancer. Towards the goal of creating a novel method of DNA hybridization detection, we report the detection of specific sequences of small oligonucleotides in a model experiment carried out in serum. The results shown here display the versatility of the DE-ATRP method in synthesizing a specific polymer structure capable of changing its physical properties in the presence of double stranded DNA. The polymer was labeled and used to detect single-stranded DNA in serum successfully.

The early detection of cancer represents one of the most promising approaches to reduce the high mortality associated with many cancers. Currently, much interest in the field of diagnostic research is focused on the detection of cancer oligonucleotide biomarkers such as mir-141 in prostate cancer.[1–4] Rapid and sensitive detection of DNA hybridization reactions is being established, but many of these methods require modification of the complementary probe in a multi-step procedure for optimal detection. In addition, many interesting DNA hybridization transducers have been reported including electrochemical and optical DNA biosensors based on conjugate polymers,[5–7] DNA-derivatized nanoparticles,[8,9] molecular beacons[10] and bioluminescence techniques.[11] However, these methods, although functional, require the modification of the polymer or amplifier properties. Even though these methods have distinct advantages over conventional procedures of oligonucleotide detection (RT-PCR, northern blotting, *etc.*[12]), specificity and reproducibility of detection are compromised. In 2004, Dore *et al.* reported that a cationic water-soluble polythiophene polymer can be used for the detection of nucleic acids at low concentrations.

Previously we have reported a deactivation enhanced atom transfer polymerization (DE-ATRP) approach, which can suppress the gelation and produce high yield "hyperbranched polymers" by the homopolymerization of multi-vinyl monomers (MVMs) even in a concentrated system.[13] In a most recent study, we realized that this DE-ATRP reaction not only provides precise control over the molecular weight and polydispersity (PDI) but also provides the potential to kinetically control the molecular architecture in the polymerization of MVMs.[14] In this study, we bring this structure to DNA detection applications through the preparation of a cationic hyperbranched polymer by the copolymerization of 2-(dimethylamino)ethyl methacrylate (DMAEMA) and ethylene glycol dimethacrylate (EGDMA) *via* the DE-ATRP approach (Scheme 1A). We demonstrate the use of a hyperbranched cationic molecule for the simple, fast and specific detection of DNA molecules without the



Scheme 1 (A) Copolymerization of DMAEMA and EGDMA *via* DE-ATRP approach leads to a hyperbranched polymer (B) which is further modified by Rhodamine B. (C) Polymer interaction with negatively charged ssDNA probe leads to an increase in fluorescence due to isolation of polymer molecules (duplex). (D) Upon complementary DNA target hybridization to the ssDNA probe, the polymer forms stronger interactions with the double stranded DNA which condenses into small nanoparticles that quench the rhodamine reducing the fluorescence intensity (triplex). (E) Adding a non-complementary target will have no effect on the fluorescence because it does not hybridize to the duplex.

[a]*Network of Excellence for Functional Biomaterials, National University of Ireland, IDA business park, Dangan, Galway, Ireland. E-mail: wenxin.wang@nuigalway.ie; Fax: +353 91 563991; Tel: +353 91 493131*
[b]*Molecular Diagnostics Research Group National University of Ireland, IDA business park, Dangan, Galway, Ireland*
[c]*School of Chemistry, Bangor University, Deiniol Road, Bangor, Gwynedd, UK. E-mail: h.tai@bangor.ac.uk*

† Electronic supplementary information (ESI) available: Description of the material, polymer synthesis and characterization methods, proton nuclear magnetic resonance and polymer/DNA complexation results. See DOI: 10.1039/c2py00516f

need for modifications of the DNA probe or target. We hypothesize that a fluorescently labeled hyperbranched polyDMAEMA-*co*-EGDMA (termed pD-*co*-E, Scheme 1B) can detect specific sequences of small DNA (18–24 nt) in serum through a change in the fluorescent signal. More specifically, detection is based on the conformational change of the polymer molecules upon binding to the ssDNA probe, producing a measurable fluorescent signal. Hybridization to the target sequence results in a significant change in fluorescence (Scheme 1D). As with any other cationic polymer, the pD-*co*-E polymer forms dense 3D-structures in solution quenching the fluorescence of rhodamine molecules.

Firstly, the formation of the polymer–ssDNA complex (termed duplex, Scheme 1C) changes the structure of the polymer due to the strong ionic interaction between the two molecules, which leads to exposure of the rhodamine fluorophores in the process. The second step involves adding the complementary sequence to the solution containing the duplex. This forms a pD-*co*-E/ssDNA probe/cDNA complex (termed triplex, Scheme 1D) from which the polymer becomes slightly dissociated. The dissociation of the polymer from the hybridized DNA allows it to regain its original structure. The principle of detection provided herein is therefore based on the amplification of the signal produced when the ssDNA probe binds to the polymer, *via* the change in the polymer's physical properties. One of the advantages of this system is that no isolation step of the duplex or triplex is required in order to obtain a signal from a relatively small sample. The method allowed for detection of specific sequences at the nanogram level in homogeneous solution but the sensitivity was significantly reduced in serum.

The hyperbranched polymer was synthesized by DE-ATRP and labeled with ethylenediamine functionalized Rhodamine B (see ESI†). Gel permeation chromatography (GPC) was used to monitor the reaction progression (Fig. S1 in ESI†). The peak area and shift were used to analyze the ratio of monomer conversion and molecular weight ($M_w = 10.6$ kDa, at 60% conversion). The low polydispersity (PDI = 1.47) indicates the formation of monodispersed, branched polymer molecules. The vinyl content, degree of branching and DMAEMA content in the polymer were determined by calculation from the ^{1}H NMR spectrum (7.1%, 11.8% and 82.1%, respectively) (Fig. S2 and eqn (S1–S6) in ESI†).

The ability of pD-*co*-E to complex (condense) DNA was assessed by UV-Visible spectroscopy (Fig. S4 in ESI†) and agarose gel electrophoresis (Fig. S5 in ESI†). The ssDNA probe illustrated in Scheme 1 is a model complementary sequence to the mir-141 (a microRNA expressed by prostate cells[15]), while the cDNA target has the same sequence as the mir-141 where the thymine groups are replaced by the uracil groups. The non-complementary target (NC-target, negative control), on the other hand, is an ssDNA sequence that is different from both sequences.

Different weight ratios of polymer to ssDNA probe were used, with no more than 1 μg of the ssDNA probe for each sample. To ensure that most of the pD-*co*-E will complex with the ssDNA probe when detecting the cDNA, equal amounts of polymer and ssDNA probe were added (*i.e.* w/w of polymer : ssDNA probe). Using the UV-Visible spectrometer, we were able to detect a decrease in ssDNA concentration after the addition of the polymer and formation of the duplex. When the cDNA target was added, an increase in absorbance was noted; however, adding the non-complementary cDNA target (termed NC-target) to the duplex solution resulted in a higher signal intensity (Fig. S6†). Even though this method can be used to differentiate between the duplex and the triplex in water, it cannot be used in biological solutions because of its non-specificity. Other proteins and DNA molecules will interrupt the signal and give a false reading which makes this method unsuitable for direct detection. This is why we decided to use a fluorescence based method of detection.

To detect the DNA hybridization, the polymer was labeled with Rhodamine B. The first set of experiments was carried out in solution where a clear difference in color sharpness between the triplex, duplex + NC-target and duplex alone could be visualized by the naked eye. Fig. 1 shows the contrast between the labeled polymer and the duplex. No color change is observed after adding the non-complementary target, but a change in color is seen when the cDNA (model microRNA) is added.

Model microRNA target (cDNA) detection was carried out by fluorescence spectroscopy. An LED light source was used for the excitation ($\lambda = 570$ nm) of the rhodamine labeled polymer and emission ($\lambda = 590$ nm) was detected. LEDs are very stable and provide a range of wavelengths with minimal background noise producing a clear signal even at low concentrations of the analyte.[16]

The fluorescence intensity of four different samples was analyzed at various concentrations (Fig. 2). After the formation of the duplex, an increase in fluorescence over the labeled polymer alone is noted even at a concentration of 8.13×10^{-5} μM of DNA. Adding the cDNA to the duplex solution resulted in a decrease in fluorescence intensity which is statistically significant down to the lowest concentration.

The response of the duplex to the NC-target is very clear, indicating that there is some sensitivity from the polymer's part towards unspecific targets. However, the change is small and becomes insignificant at lower concentrations. The change is possibly due to the binding of excess polymer with the NC-target, hence the increase in fluorescence in that sample.

When the same experiment was carried out in a serum environment (Fig. 3), the fluorescence intensity demonstrated the same changing pattern but at a significantly reduced sensitivity. This is because the experiment was carried out in a complex environment which contains large amounts of proteins and biomolecules that interfere with the signal. The results are reproducible even at a concentration of 3.25×10^{-4} mM.

However, in order to detect the endogenous microRNA that is upregulated in prostate cancer patients (present at several hundred thousand copies per microlitre in blood[17]), the detection sensitivity and specificity have to be optimized for the detection of lower concentrations. We speculate that by using a more sensitive fluorescence spectrometer, and optimizing the polymer molecular weight and charge, we will see an increase in the sensitivity of the system. The detection specificity to a single mismatch can be achieved by immobilizing the polymer onto a silica or glass surface by which removal of

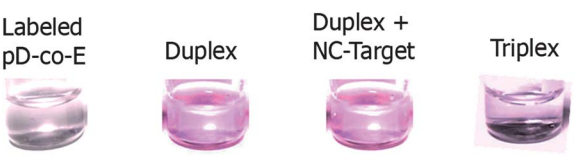


Fig. 1 Photographs of solutions (1.4×10^{-7} M, polymer concentration) containing the rhodamine labeled polymer. These images display the color changing capability of the labeled polymer when forming the duplex and triplex.

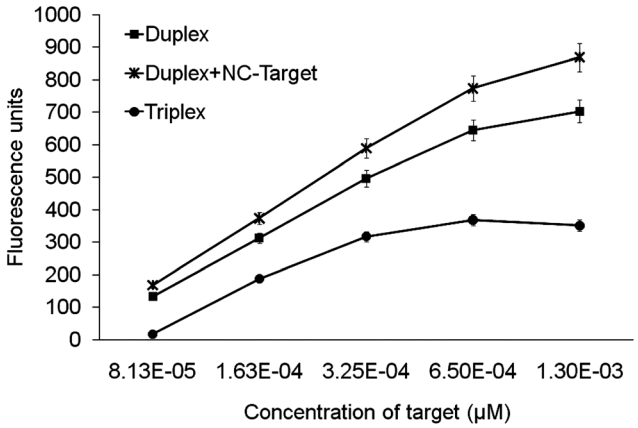


Fig. 2 Fluorescence intensity plots for the duplex, duplex + NC-target and triplex at five different concentrations. When the NC-target is added, there is a slight increase in fluorescence; however, upon adding the complementary sequence (triplex) there is a significant reduction in fluorescence further proving the change in the labeled polymer's behavior upon binding to double stranded DNA. In contrast, when the NC-target is added, the fluorescence intensity remains high ($n = 5$) (±S.D.) (for statistical significance mentioned in text $p < 0.05$).

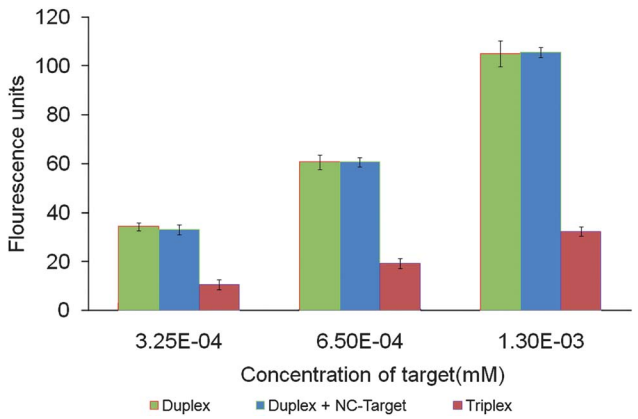


Fig. 3 Bar chart showing fluorescence intensity for the pD-*co*-E, duplex, triplex, and duplex/NC-target in serum. It is clear that even in the serum environment reproducible results are obtained allowing for easy differentiation between the complementary target and NC-target at various concentrations. No statistical significance between samples was obtained below a concentration of 3.25×10^{-4} mM ($n = 5$) (±S.D.) ($p < 0.05$).

unbound or unhybridized DNA can bring about specific sequence detection.

In conclusion, we have demonstrated successful synthesis of hyperbranched polymer using the DE-ATRP method. This hyperbranched pD-*co*-E polymer has shown exceptional physical properties demonstrated by its capability to form condensing particles that can quench the fluorescence of rhodamine molecules in a homogeneous solution and in serum. The capability of the polymer to form large complexes with double stranded DNA is what gives it the ability to quench the rhodamine molecules. The polymer and the method of detection developed herein are unique and demonstrate the applicability of the newly developed cationic, hyperbranched polymer in bioanalytical applications and specifically for the detection of DNA hybridization.

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