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Radical-generating coordination complexes as tools for rapid and effective fragmentation and fluorescent labeling of nucleic acids for microchip hybridization

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Abstract

DNA microchip technology is a rapid, high-throughput method for nucleic acid hybridization reactions. This technology requires random fragmentation and fluorescent labeling of target nucleic acids prior to hybridization. Radical-generating coordination complexes, such as 1,10-phenanthroline–Cu(II) (OP–Cu) and Fe(II)–EDTA (Fe–EDTA), have been commonly used as sequence nonspecific “chemical nucleases” to introduce single-strand breaks in nucleic acids. Here we describe a new method based on these radical-generating complexes for random fragmentation and labeling of both single- and double-stranded forms of RNA and DNA. Nucleic acids labeled with the OP–Cu and the Fe–EDTA protocols revealed high hybridization specificity in hybridization with DNA microchips containing oligonucleotide probes selected for identification of 16S rRNA sequences of the *Bacillus* group microorganisms. We also demonstrated cDNA- and cRNA-labeling and fragmentation with this method. Both the OP–Cu and Fe–EDTA fragmentation and labeling procedures are quick and inexpensive compared to other commonly used methods. A column-based version of the described method does not require centrifugation and therefore is promising for the automation of sample preparations in DNA microchip technology as well as in other nucleic acid hybridization studies.

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DNA microchips, consisting of arrays of immobilized DNA probes, allow large numbers of hybridization reactions to be run simultaneously. DNA microchip technology is thus an extremely high-throughput method for nucleic acid hybridization reactions.

DNA microchip protocols require random fragmentation and fluorescent labeling of target nucleic acids prior to hybridization. Sequence nonspecific fragmentation is necessary to reduce the size of the nucleic acid molecules and to destroy their three-dimensional structure, making the molecule more accessible for hybrid-

ization. Fragmentation also allows different regions of the target molecules to hybridize independently to each of the immobilized oligonucleotides [1–9]. Treatment of RNA with divalent ions [1–7,10] or alkali [11] and treatment of DNA with acid [11,12] or nucleases [5,8,9] are currently the most popular methods for fragmentation of nucleic acids.

Although fluorescent labeling is less sensitive than radioactive labeling, it offers several advantages for hybridization detection. Fluorescent dyes do not pose radiation hazards, and thus their use and disposal are less problematic. Moreover, fluorescent labels can be detected in real time with high resolution [13]. Several different protocols for fluorescent labeling of DNA and RNA utilizing enzymatic [1,2,5–9,14–23] or chemical [6,11,14] methods have been described. However, all of

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these methods are time consuming (on the order of hours) and often are rather expensive (requiring labeled or biotinylated triphosphate nucleotides and enzymes). These methods of nucleic acid labeling with polymerases are useful when amplification is necessary. However, when a considerable amount of cells may be grown or collected, as in gene expression studies [4–6,15,17,19,21–23], direct labeling and fragmentation of nucleic acids are more reasonable. For the same reason, direct labeling is more useful in bacteria detection experiments, where naturally amplified rRNA has been targeted [7,24–26]. Moreover, direct labeling is advantageous as it eliminates the bias inherent in amplification [27].

The DNA microchips utilized in this study consisted of an array of polyacrylamide gel pads affixed to a glass slide [12,28]. These gel pads served as three-dimensional supports for the immobilization of oligonucleotide probes targeting the 16S rRNA of several members of the *Bacillus* group of microorganisms.

Oxidants with free radical characteristics are well known tools for DNA and RNA modifications [29]. Redox-active coordination complexes such as (OP)–Cu and Fe–EDTA are commonly used as “chemical nucleases” to introduce single-strand breaks in nucleic acids [30–32]. It is also known that treatment of DNA or RNA with an oxidizing agent or free radical results in the production of a reactive abasic site on the nucleic acid molecule [29]. We have previously used this sort of modification, obtained after purine methylation [33,34], or nucleic acid treatment with radical-generating coordination complexes [34,35], for crosslinking of proteins to nucleic acids through the amino groups or through the imidazole rings of histidines. In this study we attempted to utilize the reactive abasic sites generated by free radicals for the direct crosslinking of nucleic acids to fluorophores containing amino groups, or for the indirect crosslinking of nucleic acids to amino-reactive fluorophores through ethylenediamine (EDA).²

The goals of the current study were to determine if the radical-generating coordination complexes OP–Cu and Fe–EDTA could be effectively used for both nucleic acid fragmentation and labeling with fluorescent dyes as part of an overall DNA microchip protocol. As a result, an inexpensive method of nucleic acid fragmentation and labeling with the aid of radical-generating coordination complexes that provides sample preparation in a minutes instead of hours has been developed.

Materials and methods

RNA isolation

Total RNA was isolated from frozen cell pellets of *Bacillus cereus* 9620 and *Bacillus thuringiensis* 4042B. RNA was isolated by bead beating, phenol–chloroform extraction, and ethanol precipitation as previously described [27]. For isolation of single-stranded RNA, a 300-nucleotide fragment was transcribed from a PCR-amplified DNA fragment from the first exon I of the human mu opioid receptor and purified as previously described [36].

DNA preparation

16S rDNA was synthesized by PCR amplification of bulk DNA from *B. cereus* T or *Bacillus cereus* 3329 with AmpliTaq DNA polymerase (Ambion, Austin, TX) using 11F (GTTTGATCCTGGCTCAG) and 1512R (RGTGAGCTRTTACGC, where R = A or G) primers.

Magnesium–sodium periodate fragmentation and labeling of RNA: direct protocol

RNA (10–20 µg) and DEPC treated H₂O were combined in total volume of 19 µl and preheated at 95 °C for 5 min. One microliter of 1.2 M MgCl₂ was added and the reaction solution was heated at 95 °C for 40 min. Phosphatase treatment was carried out by addition of 3 µl 10X alkaline phosphatase buffer (Promega, Madison, WI) containing 0.5 M Tris–Cl, pH 9.3, 10 mM MgCl₂, 10 mM spermine, and 0.2 µl alkaline phosphatase (1 u/µl) (Promega) and heating at 37 °C for 30 min. Oxidation was conducted by addition of 6.5 µl of 100 mM sodium periodate and incubation at room temperature for 20 min.

Direct labeling was carried out by the addition of 3.5 µl of 100 mM freshly prepared and filtered Lissamine rhodamine B ethylenediamine (LissRhod) (Molecular Probes, Eugene, OR) and 1.65 µl of 1 M HEPES (pH 7.5) and heating at 37 °C for 1 h. Reduction was conducted by addition of 6.7 µl of 200 mM sodium cyanoborohydride and incubation at room temperature for 30 min. Labeled RNA was precipitated by addition of 15 vol of 2% lithium perchlorate in acetone and storage at –20 °C for 20 min. After centrifugation in an Eppendorf 5415C microcentrifuge at 14,000 rpm (~14,000g) for 5 min, RNA pellets were washed twice with acetone and dried at 55 °C for 10 min.

Butanol treatment

Excess LissRhod was removed from RNA by butanol treatment: RNA pellets were suspended in 300 µl DEPC-treated H₂O, and samples were concentrated to 60 µl by

² Abbreviations used: OP, 1,10-phenanthroline or o-phenanthroline; EDA, ethylenediamine; DEPC, diethyl pyrocarbonate; LissRhod, Lissamine rhodamine B ethylenediamine; PCR, polymerase chain reaction; TexRed, Texas Red.

removal of water with butanol. Treatment was repeated three or four times until butanol was free of color. RNA was precipitated in 15 vol of 2% LiClO₄ in acetone at –20°C for 20 min. After centrifugation at 14,000 g for 5 min, RNA pellets were washed twice with acetone, dried at 55°C for 10 min, and suspended in 10–20 µl DEPC-treated H₂O.

OP–Cu labeling and fragmentation of RNA: direct protocol

To maintain anaerobic conditions, all reagents were bubbled with argon for 15 s before use, and the reaction solutions were bubbled with argon for 15 s between each step. Total volume of labeling-fragmentation reaction was 100 µl. RNA (10–20 µg), 20 µl of 100 mM sodium phosphate (pH 7.0), urea (if necessary), and DEPC-treated H₂O were combined and bubbled with argon. After addition of stock solutions of *o*-phenanthroline hydrochloride monohydrate (OP) (Fluka, Ronkonkoma, NY), CuSO₄ × 5 H₂O (Cu), and 1 µl of 100 mM freshly prepared and filtered LissRhod, the solution was bubbled with argon and preheated for 3 min. (Stock solutions of 150 mM OP and 15 mM Cu may be stored for at least 1 year at room temperature.) The solution was again bubbled with argon and H₂O₂ was added. The reaction solution was then bubbled with argon and heated for 10–30 min. Reaction was stopped by addition of 2 µl 0.5 M EDTA and incubation in a room temperature water bath for 1 min.

Reduction was carried out by addition of 200 mM sodium cyanoborohydride to 20 mM and incubation at room temperature in the dark for 30 min. RNA was precipitated in 96% ethanol/0.4 M sodium acetate at –80°C for 20 min. After centrifugation at 14,000 g for 5 min, RNA pellets were washed twice with ethanol. Excess LissRhod was removed from RNA by butanol treatment as described above and RNA pellets were suspended in 10–20 µl DEPC H₂O.

Fe–EDTA labeling and fragmentation of RNA: direct protocol

To maintain anaerobic conditions, all reagents were bubbled with argon as described for the OP–Cu direct protocol. The stock solution of Fe–EDTA complex consisted of 0.5 M EDTA and 0.25 M ammonium iron (II) sulfate. The total volume of the labeling-fragmentation reaction was 100 µl. RNA (10–20 µg), 20 µl 100 mM sodium phosphate, pH 7.0, DEPC treated H₂O, urea (if necessary), and the Fe–EDTA complex were combined and bubbled with argon. After bubbling, 100 mM freshly prepared and filtered LissRhod was added to final concentration of 1 mM and solution was bubbled with argon and preheated for 3 min at 95°C. H₂O₂ and sodium ascorbate (NaAsc) were added.

The reaction solution was again bubbled with argon and then heated to 95°C for 10–30 min. Reaction was stopped by addition of 10 µl 1 M thiourea and incubation in a room temperature water bath for 1 min. Reduction, ethanol precipitation, excess dye extraction, and RNA pellets dilution were performed as described in OP–Cu direct protocol.

Indirect RNA-labeling protocol

For indirect labeling with magnesium–sodium periodate, the OP–Cu or the Fe–EDTA method, LissRhod was replaced in the fragmentation protocol by 10 µl 0.5 M ethylenediamine. After reduction with NaCNBH₃ and ethanol precipitation, the RNA pellet was dissolved in 100 µl 100 mM sodium carbonate (pH 9.0). The mixture was transferred to freshly opened ampule containing 1 mg Texas Red sulfonyl chloride (TexRed) (Molecular Probes), precooled with ice, and incubated in the dark on ice overnight. The reaction was stopped by adding 40 µl 1 M acetic acid and the mixture was diluted with 200 µl 100 mM sodium acetate (pH 4.0). Excess TexRed was removed from RNA by butanol treatment as described above. RNA pellets were suspended in 10–20 µl DEPC H₂O.

Silica minicolumn RNA-labeling protocol

Samples of labeled and fragmented nucleic acids were obtained from *B. mycoides str.* NCTC8096 cells as described earlier [37] with some modifications. Five microliters of silica suspension was applied to a 25-mm-long sterile disposable centrifuge device containing a polysulfone filter with a diameter of 6.5 mm and a pore size of 0.2 µm (Whatman, Fairfield, NJ) and centrifuged in an Eppendorf 5415C microcentrifuge for 30–60 s at 10,000 rpm (~8400g). Silica in the column was washed once with 100 µl of H₂O (pipetting of silica was followed by centrifugation under the same conditions). Cells (2 × 10⁸) were pretreated in 25 µl of 100 mg/ml lysozyme at 37°C for 5 min, diluted with 550 µl of a mixture (9:4) of L and B buffers [37], and applied to the silica minicolumn. All further operations performed previously by syringe [37] were conducted by centrifugation for 30–60 s at 8400g. All manipulations with a silica minicolumn, except the labeling-fragmentation step, were carried out at room temperature. The column was washed with 100 µl of L:B buffer mixture (9:4) (twice), 100 µl of 75% ethanol (twice), and 100 µl of 100% ethanol (twice). The silica column containing nucleic acids was sealed at the bottom with a cap from a 1.5-ml microcentrifuge tube and preheated in a sand bath at 95°C for 3 min to remove the traces of ethanol. A freshly prepared labeling cocktail without H₂O₂ (16.5 mM OP, 1.65 mM Cu, 1.1 mM LissRhod, 22 mM sodium phosphate, pH 7.0) was centrifuged for 30 s at 8400g to

remove insoluble admixtures of the dye and 90 μ l of it was preheated for 30 s at 95 °C. Ten microliters of 1 M H₂O₂ was added to the cocktail immediately before application of 30 μ l of hot cocktail to the preheated minicolumn. The minicolumn was sealed at the top to prevent evaporation and was incubated in the sand bath for 1 min at 95 °C. The reaction was stopped by adding 4 μ l of freshly prepared stop solution (2.6 M sodium acetate, pH 5.2, and 70 mM EDTA) and 100 μ l of cold 100% ethanol into the bath column. After a 5-min incubation at room temperature, nucleic acids were precipitated on the column by centrifugation for 1 min at 8400g. Excess fluorescent label was removed by successive washing of the column with 100 μ l of 75 and 100% ethanol. For elution of labeled product, the silica in the column was suspended (twice) with 30 μ l of 10 mM sodium carbonate, pH 8.5, and the column was sealed from the top, placed into the microcentrifuge tube, incubated for 2 min at 95 °C, and centrifuged for 30 s at 8400g. Yield of labeled nucleic acids was about 15–20 μ g.

DNA labeling

OP–Cu and the Fe–EDTA protocols were run using procedures described above for direct and indirect labeling of RNA. 16S rDNA was produced by PCR amplification of bulk DNA from *B. cereus* T. The direct labeling–fragmentation reaction was performed with 15 mM OP, 1.5 mM Cu, 100 mM H₂O₂, 1 mM LissRhod at 45 °C for 30 min under argon bubbling and was followed by reduction with 20 mM NaCNBH₃. The indirect reaction was carried out with 1.5 mM OP, 0.15 mM Cu, 10 mM H₂O₂, 50 mM EDA at 45 °C for 30 min under argon bubbling and was followed by reduction with 20 mM NaCNBH₃ and labeling with 16 mM Tex-Red as described in indirect RNA-labeling protocol.

The rapid OP–Cu protocol was run under the following conditions: labeling cocktail freshly prepared without argon bubbling and containing 15 mM OP, 1.5 mM Cu, 10 mM H₂O₂, 20 mM NaCNBH₃, 1 mM LissRhod, 20 mM sodium phosphate, pH 7.0 was mixed with 5–10 μ g DNA and incubated in total volume 100 μ l at 95 °C for 10 min. The reaction was stopped and excess dye was removed as described in standard direct protocol for RNA.

The rapid Fe–EDTA protocol was run with DNA with the following specific conditions: 3 mM Fe, 10 mM H₂O₂, 1 mM LissRhod at 95 °C for 10 min. The NaCNBH₃ reduction step and argon bubbling were excluded.

Gel electrophoresis

Fragmented and labeled RNA samples were analyzed by polyacrylamide gel electrophoresis under denaturing

conditions (8 M urea) using 8, or 20% polyacrylamide gel (acrylamide:bis-acrylamide, 1:19) [38]. Each well received 3 μ l of RNA and 17 μ l of gel loading buffer (Ambion, Austin, TX). Fluorescently labeled fragments were visualized after electrophoresis directly using 312 nm Transilluminator FBTI 88 (Fisher Scientific, Pittsburgh, PA). After ethidium bromide staining of the same gel, both labeled and unlabeled fragments were detected with the same transilluminator.

Computer selection of genus- and species-specific oligonucleotide probes

For selection of genus-specific probes, the 16S rRNA sequence from a specific microorganism belonging to this genus was used to create a set of all possible 20 b oligonucleotide probes (the set consisted of L-19 oligonucleotides, where L denotes the length of the entire 16S rRNA sequence). Each potential probe was tested against all available 16S rRNA sequences (GenBank and RDP) by a function that estimates the relative duplex stability according to the number and position of mismatches. If the 16S rRNA of any microorganism that did not belong to the genus of interest formed stable duplexes with any oligonucleotide considered as a probe for the microchip, this oligonucleotide was excluded from the list of probes. A similar procedure was carried out for the selection of species-specific probes. A final set of 15 oligonucleotide probes each approximately 20 b in length (Table 1) was selected for application to the DNA microchip.

DNA microchip manufacturing and design

The selected oligonucleotides (Table 1) were synthesized and applied on the microarray matrix, containing 100 \times 100 \times 20 μ m polyacrylamide gel pads affixed to a glass slide as described earlier [37,39].

Hybridization with microchips and analysis of hybridization data

Labeled nucleic acids were hybridized with microchips as described previously [37]. After washing the microchip was imaged using a fluorescence microscope, CCD camera, and WinView software as previously described [40]. The fluorescent intensity of each gel element was quantified from the WinView image using LabView software customized by our laboratory. The score for each gel element was calculated by subtracting the averaged fluorescent intensity of the area immediately surrounding the gel element (i.e., the background) from the averaged fluorescent intensity of the entire area of the gel element. In order to compare experimental treatments, the hybridization signal for each experimental treatment was calculated by averaging the scores

Table 1
Oligonucleotide probes on DNA microchips

Probe	Length	Sequence	16S rRNA 5'-end location ^a	Target
1	17	ACG GGC GGT GTG TRC AA	1400	Universal (all life, U2) ^b
2	18	GWA TTA CCG CGG CKG CTG	529	Universal (all life, U1) ^b
3	18	TGC CTC CCG TAG GAG TCT	345	Eubacteria ^b
4	17	ACC GCT TGT GCG GGC CC	938	Eubacteria ^b
5	20	CGA AGC CGC CTT TCA ATT TC	203	<i>B. cereus</i> group
6	20	CAA CTA GCA CTT GTT CTT CC	455	<i>B. cereus</i> group (BCG2)
7	20	TGT CAC TCT GCT CCC GAA GG	1038	<i>B. cereus</i> group
8	20	CGG TCT TGC AGC TCT TTG TA	1257	<i>B. cereus</i> group (BCG1)
9	23	ATG CGG TTC AAA ATG TTA TCC GG	175	<i>B. cereus</i> str. 9620, ^c T ^c and <i>B. thuringiensis</i> str. 4042B ^c
10	20	TTC GAA CCA TGC GGT TCA AA	186	<i>B. cereus</i> str. 9620, ^c T ^c and <i>B. thuringiensis</i> str. 4042B ^c
11	20	TTC GAA CTA TGC AGT TCA AA	186	<i>B. mycoides</i> str. 6462m ^c
12	23	CAA TTT CGA ACT ATG CAG TTC AA	187	<i>B. mycoides</i> str. 6462m ^c
13	20	ACA GAT TTG TGG GAT TGG CT	1285	<i>B. subtilis</i> group (BSG2)
14	19	ATT CCA GCT TCA CGC AGT C	1333	<i>B. subtilis</i> group (BSG1)
15	20	CGG TTC AAA CAA CCA TCC GG	175	<i>B. subtilis</i> spp. (BS1)
16	20	AAG CCA CCT TTT ATG TTT GA	201	<i>B. subtilis</i> spp. (BS2)

^a Locations indicated for *B. cereus* NCTC9620 16S rRNA.

^b See [24].

^c Belongs to *B. cereus* group.

for the four oligonucleotide probes targeting the cereus group, probes 5–8 (Table 1). The intensity of the hybridization signal was used to assess the effectiveness of different reaction parameters for the fragmentation and labeling procedures.

Hybridization signals reported represent average signals calculated from replicate data. Within each experiment, treatments were replicated from 2 to 4 times, and the variation in hybridization signals for each treatment was less than 20%.

Results and discussion

Putative chemistry of nucleic acid modification, fragmentation, and dye crosslinking with OP–Cu and Fe–EDTA

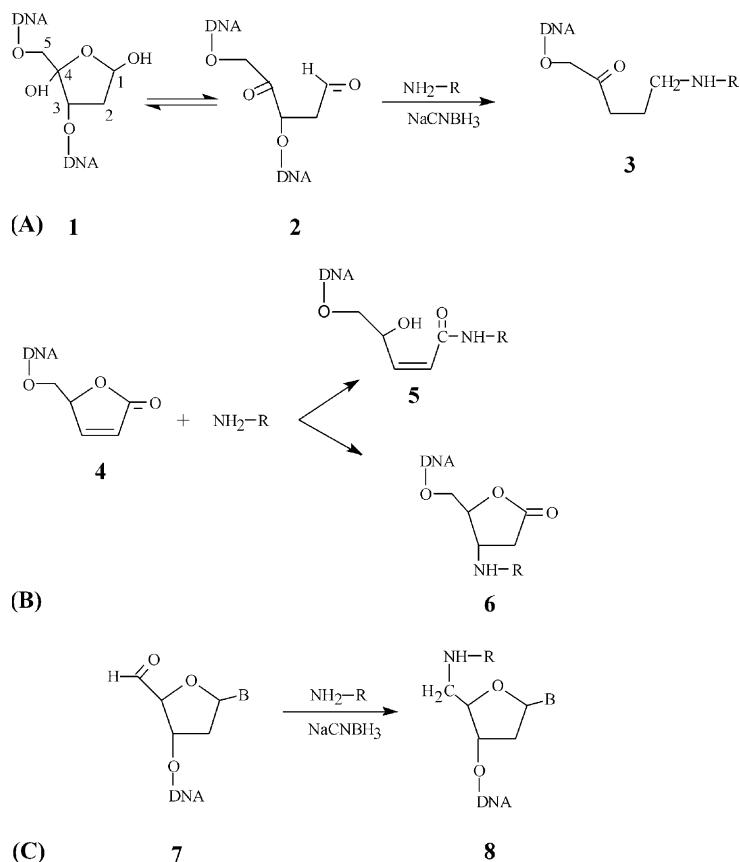
The interactions of OP–Cu and Fe–EDTA with DNA have been extensively studied. OP–Cu binds to double-stranded DNA in the minor groove and in the presence of hydrogen peroxide promotes DNA cleavage by the abstraction of a hydrogen atom [32]. Five carbon atoms (Scheme 1A,1) of the DNA sugar residue have a total of seven hydrogen atoms, which are available for abstraction by an oxidizing agent [32]. The main pathway of DNA cleavage by OP–Cu is H-1' abstraction, but OP–Cu can also cleave DNA by a minor pathway that begins with abstraction of H-4' [30,32]. The degradation of DNA by OP–Cu has some slight sequence specificity [41,42].

The Fe–EDTA complex is negatively charged, and thus it does not interact directly with the DNA molecule. Instead, the Fe-EDTA complex, in the presence of

hydrogen peroxide, produces hydroxyl radicals which have no charge and are therefore able to diffuse freely into the DNA molecule. Hydroxyl radicals are able to abstract any of the hydrogen atoms from the carbon atoms within the deoxyribose residues of B-form DNA, but abstraction from the 4'- and 5'-positions are the predominant pathways. Preference for individual hydrogen atoms was found to be H-5' > H-4' > H-2' = H-3' > H-1', which correlates with the accessibility of the individual hydrogen atoms to a solvent [32]. Hydroxyl radicals have no specificity for cleavage of DNA at a particular nucleotide [32,43,44].

The proposed mechanisms of DNA degradation via hydrogen atom abstraction can be influenced by the presence of oxygen [32]. Most of these reactions, with the exception of H-4' abstraction under aerobic conditions, result in nucleobase release with the formation of intermediates which may react with primary amines and thus may be used for DNA crosslinking with amino conjugates of fluorescent dyes (Scheme 1). Alternatively, these intermediates may also be crosslinked with EDA and subsequently labeled with amino-reactive fluorophores [45].

H-4' abstraction under anaerobic conditions results in nucleobase release with the production of a hemiacetal intermediate that is in equilibrium with the aldehyde form of deoxyribose **2** (Scheme 1A)[32]. This aldehyde group may be attacked by a nucleophilic moiety (such as a primary amine or a hydrazide), creating a reversible covalent bond (Schiff base), and the resultant imine undergoes spontaneous conversion in which the 3'-phosphodiester bond is cleaved by the mechanism of β -elimination. In this way, the crosslinking of amine or



Scheme 1. Putative mechanisms of dye crosslinking to intermediates in radical-mediated DNA modification pathways. R, fluorescent dye or EDA moiety. (A) anaerobic H-4' abstraction pathway, (B) H-1' abstraction pathway, (C) H-5' abstraction pathway.

hydrazine derivatives of the fluorescent dyes to the modified DNA can occur at the same time as the fragmentation. This sort of chemistry has been suggested earlier by our group as an explanation for the canonical Mirzabekov DNA–protein crosslinking reaction [33]. After the fragmentation and crosslinking, reduction of the Schiff base with sodium cyanoborohydride is desirable for production of a stable covalent bond **3**, thus preventing removal of the crosslinked dye by β -elimination [33].

In contrast to the H-4' anaerobic pathway, H-4' abstraction under aerobic conditions leads to the complete splitting of deoxyribose [32], and the intermediates of this pathway may not be used for labeling with aminoconjugates. Because the proposed H-4' abstraction pathways indicate that amine crosslinking might be less effective under aerobic conditions, we initially used anaerobic conditions in this study.

Another DNA intermediate that may be used for labeling with amino derivatives of fluorescent dyes is lactone **4** (Scheme 1, B). This lactone has been identified as an intermediate in both aerobic and anaerobic H-1' abstraction pathways [32]. Reaction of this lactone with a primary amine would lead to products **5** and **6**.

The H-5' abstraction pathway under both aerobic and anaerobic conditions may result in the production of an oligonucleotide 5'-aldehyde **7** (Scheme 1C)[32]. This aldehyde may interact with amines through the formation of a Schiff base in the same manner as described for the H-4' anaerobic pathway (see Scheme 1A). For this labeling reaction, the presence of sodium cyanoborohydride in the reaction buffer or immediate sodium cyanoborohydride treatment following Fe–EDTA treatment is desirable for fast Schiff base reduction and production of stable covalent complex **8**.

Radicals generated with OP–Cu and Fe–EDTA effectively attack both DNA and RNA. The specific mechanism of RNA fragmentation by radical-mediated oxidation is not well understood, but treatment of identical RNA and DNA sequences with OP–Cu complexes linked to carrier oligonucleotides has demonstrated that both the cutting sites and the kinetics of fragmentation are similar for RNA and DNA [46]. In addition, we have previously demonstrated that OP–Cu effectively crosslinks histones both to ribooligonucleotides and to deoxyribooligonucleotides as well as to DNA in bulk chromatin in vitro and in vivo [35]. However, there are some unexpected differences in the reactions of OP–Cu with DNA and RNA. For example,

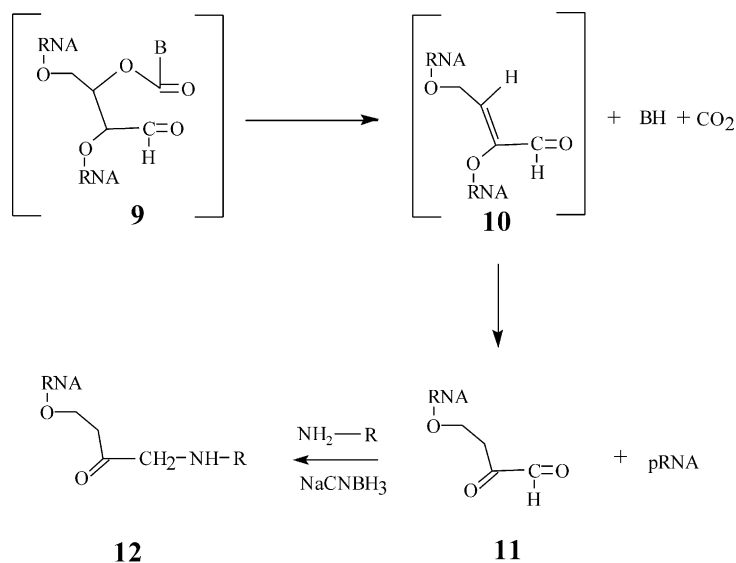
because OP intercalates into the minor groove of B-form DNA, OP–Cu cleaves dsDNA more efficiently than ssDNA. However, for RNA, OP–Cu degrades loop regions more quickly than duplex regions [47,48]. This difference in the reactions for DNA and RNA may be due to steric effects.

Another possible difference in the reaction of OP–Cu with DNA and RNA is that the putative intermediate **9** (Scheme 2), suggested for the RNA H-1' abstraction pathway [49], may serve as a substrate for crosslinking with primary amines that may result in stable products **12**.

Linking the dye to the end of the nucleic acid fragment is more useful than having the dye randomly localized along the fragment because having the dye on the end of the fragment causes minimal steric interference during subsequent hybridization. Radical-mediated labeling seems to be an effective method for placing the majority of the dye on the ends of the nucleic acid fragments. According to the proposed chemistry, radical-mediated labeling results in the crosslinking of the fluorescent dye to the 5'- or 3'-end of the nucleic acid strand (see Schemes 1 and 2). In addition, in our recent studies of OP–Cu-mediated protein–DNA crosslinking [35], the crosslinking occurred at the 5'-end or the 3'-end of the DNA molecule in approximately 90% of the crosslinked complexes, and crosslinking occurred randomly along the DNA fragment in approximately 10% of the complexes (Gavin and Bavykin, manuscript in preparation).

One potential drawback to the application of hydroxyl radical fragmentation to DNA microchip

technology is the possibility of damage to nucleobases. A number of studies have indicated that a considerable part of the DNA damage inflicted by hydroxyl radicals occurs on nucleobases [29,50]. This damage to nucleobases could negatively impact the ability of nucleic acids labeled with radical-generating coordination complexes to hybridize with oligonucleotide probes. However, most nucleobase damage does not modify functional groups involved in Watson–Crick interactions [29,50,51]. Therefore, nucleobase damage should not affect the hybridization capability of the modified bases. In addition, earlier studies have demonstrated that in the presence of excess reductants, such as Fe(II) or sodium ascorbate, oxidized nucleobases may actually be converted back to their native state [52]. This explains why radiation treatment reveals predominantly nucleobase damage but Fenton chemistry of transition metals yields a major amount of sugar hydrogen atom abstraction and direct strand scission [29]. This explanation is not complete, however, because it has been shown that nucleobase modification by OP–Cu complexes requires the presence of a reducing agent such as ascorbic acid, mercaptoethanol, or hydrogen peroxide [51]. Within our fragmentation and labeling protocols, nucleobase oxidation may also impact dye crosslinking. In general, oxidation of nucleobases removes electron density from the heterocycles, which makes the phosphoglycosidic bonds more labile and which results in the formation of abasic sites [29]. In our reactions, these abasic sites, in addition to abasic sites that appear as a result of sugar hydrogen abstraction, may be involved in dye or EDA crosslinking (Scheme 1).



Scheme 2. Putative mechanisms of dye crosslinking to intermediates in radical-mediated RNA modification pathways R, fluorescent dye or EDA moiety. A and B, two possible H-1' abstraction pathways.

Mechanisms of direct and indirect labeling

For the direct labeling protocol, the active aldehyde, lactonic, or oxycarbamide groups produced within the sugar moiety may be directly crosslinked with amine or hydrazine conjugates of fluorescent dyes. We used the fluorescent dye LissRhod for direct labeling of both RNA and DNA. The resultant Schiff base was subsequently reduced with sodium cyanoborohydride.

In the first stage of the indirect labeling protocol, EDA was crosslinked to the nucleic acids instead of the fluorescent dye, forming a Schiff base. In the next stage, the amino-modified nucleic acid may be crosslinked to fluorophores containing amino-reactive groups, such as sulfonyl chlorides, isothiocyanates, succinimidyl conjugates, fluorescamine, aromatic dialdehydes (such as OPA, NDA, or ADA) or ATTO-TAG reagents (for review see [45]). We used TexRed for indirect labeling of both RNA and DNA. The indirect labeling protocol is especially useful for dyes that are unstable in the presence of radicals, since the labeling step occurs after the radical fragmentation reaction has been completed.

OP–Cu protocol

Screening of several different reactant concentrations demonstrated that the most effective concentrations for indirect labeling with OP–Cu at 45 °C for 30 min were 1.5 mM OP/0.15 mM Cu/10 mM H₂O₂. These concentrations resulted in effective RNA-labeling (Fig. 1A, c), RNA fragments between approximately 50 and 100 b in length (Fig. 1B, c), effective hybridization (Fig. 2C), as well as a strong hybridization signal of 1700 μ/μg/s. Increasing the hydrogen peroxide concentration 10 times to 100 mM resulted in strong RNA-labeling (Fig. 1A, b) but, due to excessive RNA digestion, it also resulted in shorter fragments (Fig. 1B, b), selective degradation of some rRNA regions with subsequent loss of the hybridization signals from some probes (Fig. 2B), and a decrease in the hybridization signal to 930 u/μg/s. Increasing the concentrations of all reagents 10 times to 15 mM OP/1.5 mM Cu/100 mM H₂O₂ resulted in almost complete degradation of the RNA (Fig. 1B, a) and a further decrease in the RNA labeling (Fig. 1A, a), loss of almost all hybridization

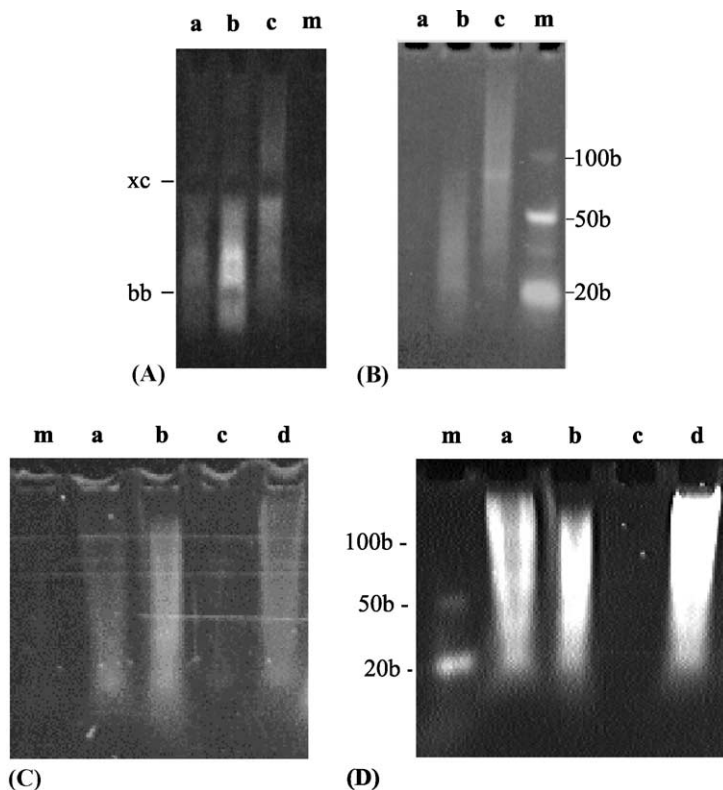


Fig. 1. Direct and indirect labeling and fragmentation of bulk RNA. Electrophoresis of labeled and fragmented *B. thuringiensis* 4042B rRNA was performed under denaturing conditions on 8% polyacrylamide gel. (A and B) Indirect labeling and fragmentation via OP–Cu at 45 °C for 30 min. (A) fluorescence image, (B) ethidium bromide-stained gel image. (a) 15 mM OP, 1.5 mM Cu, 100 mM H₂O₂; (b) 1.5 mM OP, 0.15 mM Cu, 100 mM H₂O₂; (c) 1.5 mM OP, 0.15 mM Cu, 10 mM H₂O₂; (m) single stranded marker oligonucleotides, fragment length in nucleotides (b) is indicated on the right. xc, xylene cyanol, bb, bromophenol blue, (C and D) Direct labeling and fragmentation via Fe–EDTA at 95 °C for 10 min. (C) Fluorescence image and (D) ethidium bromide-stained gel image; m, single-stranded marker oligonucleotides, (a) 15 mM Fe, 30 mM EDTA, 10 mM H₂O₂, 1 mM NaAsc, (b) 1.5 mM Fe, 3 mM EDTA, 10 mM H₂O₂, 1 mM NaAsc, (c) 150 mM Fe, 300 mM EDTA, 100 mM H₂O₂, 1 mM NaAsc, (d) 15 mM Fe, 30 mM EDTA, 10 mM H₂O₂, 10 mM NaAsc.

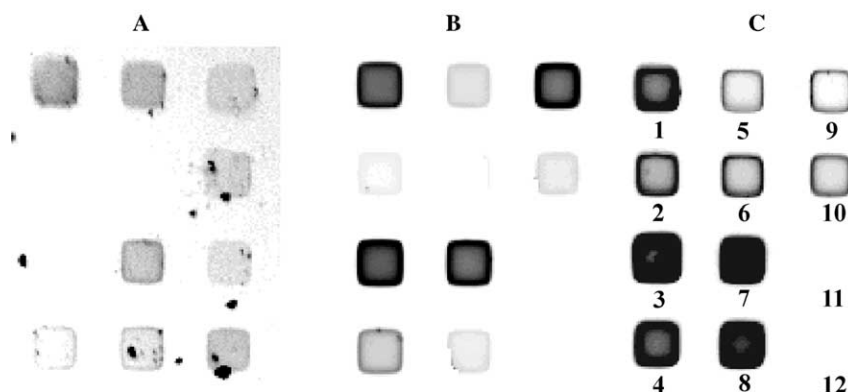


Fig. 2. Hybridization of microchip with bulk *B. thuringiensis* 4042B RNA fragmented and labeled for 30 min at 45 °C via OP–Cu indirect labeling protocol with 16 mM TexRed and: (A) 15 mM OP, 1.5 mM Cu, 100 mM H₂O₂, (B) 1.5 mM OP, 0.15 mM Cu, 100 mM H₂O₂, (C) 1.5 mM OP, 0.15 mM Cu, 10 mM H₂O₂. Numbers indicate probes listed in Table 1.

(Fig. 2A), and a decrease in the hybridization signal to 50 u/μg/s.

For the direct OP–Cu-labeling procedure, screening experiments performed at 45 °C for 30 min demonstrated that the optimal reactant concentrations were 15 mM OP/1.5 mM Cu/100 mM H₂O₂. These concentrations produced strong RNA labeling and a strong hybridization signal of 949 μ/μg/s (Table 2, Fig. 3).

The optimal direct and indirect OP–Cu protocols resulted in similar hybridization patterns (Fig. 3) and both produced strong hybridization signals (Table 2). However, the optimal reactant concentrations for the direct labeling protocol were 10 times higher than the optimal concentrations for the indirect labeling protocol.

Screening experiments run with various reaction times, reagent concentrations, and reaction temperatures (data not shown) also demonstrated that the time of the direct OP–Cu reaction could be effectively

shortened to 10 min if the temperature was raised to 95 °C and the reaction conditions were set to 15 mM OP/1.5 mM Cu/10 mM H₂O₂. These parameters generated a strong hybridization signal of 1150 μ/μg/s.

We studied the effect of urea on RNA labeling and fragmentation with the direct OP–Cu method at 45 and at 95 °C. We found that at 45 °C, the addition of 3.5 M urea resulted in a 43% increase in labeling of *B. cereus* NCTC9620 bulk RNA at concentration of hydrogen peroxide 10 times lower than the optimal conditions shown in Table 2. In contrast, when the OP–Cu reaction was run at 95 °C, the addition of 3.5 M urea and a 10-fold reduction in hydrogen peroxide concentration resulted in a reduction in RNA labeling by a factor of 2.9. This difference in the effect of urea at different reaction temperatures may indicate that 95 °C or 3.5 M urea at 45 °C are adequate to disrupt the RNA secondary structure. This results suggested that urea is not necessary when the fragmentation and labeling reactions are

Table 2

Comparison of hybridization signals of direct and indirect RNA^a labeling via Mg²⁺, OP–Cu, and Fe–EDTA methods

Method		Fragmentation reaction conditions		Reactant concentrations	Hybridization signal ^b (u/μg/s)
		Temperature (°C)	Time (min)		
Mg ²⁺ –NaIO ₄	Direct	95	40	60 mM Mg ²⁺	844
Mg ²⁺ –NaIO ₄	Indirect	95	40	60 mM Mg ²⁺	1142
Fe–EDTA	Direct	95	10	1.5 mM Fe 3 mM EDTA 10 mM H ₂ O ₂	870
Fe–EDTA	Indirect	95	10	1.5 mM Fe 3 mM EDTA 1 mM H ₂ O ₂	1094
OP–Cu	Direct	45	30	15 mM OP 1.5 mM Cu 100 mM H ₂ O ₂	949
OP–Cu	Indirect	45	30	1.5 mM OP 0.15 mM Cu 10 mM H ₂ O ₂	1309

^a *B. cereus* 9620 bulk RNA.

^b Average of signal from probes 5, 6, 7, 8.

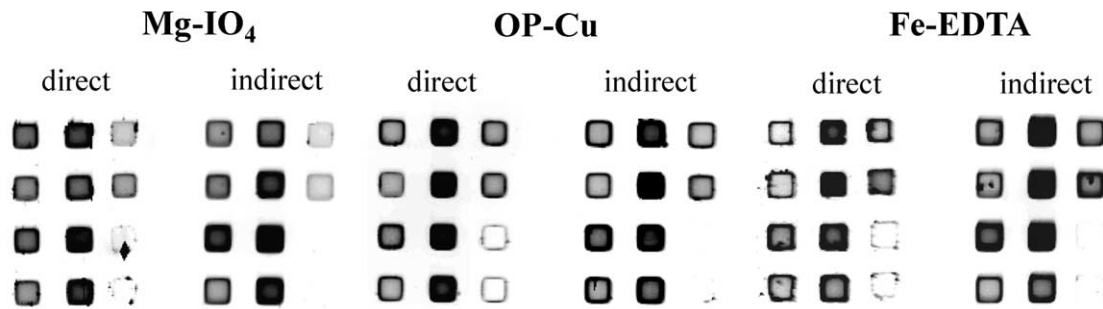


Fig. 3. Comparison of hybridization images for bulk *B. thuringiensis* 4042B RNA treated by different fragmentation and labeling methods: Magnesium sodium periodate method (Mg-IO₄), 1,10-phenanthroline-Cu(II) method (OP-Cu), and Fe(II)-EDTA method (Fe-EDTA). For probe description and positioning; see Fig. 2. Hybridization conditions are described in Table 2.

run at 95 °C. Unlike RNA labeling at 45 °C (Fig. 2B), we did not find selective degradation of some regions in 16S rRNA in samples with excessive RNA digestion after labeling at 95 °C (data not shown). This finding also suggests complete RNA unfolding in labeling reaction at 95 °C without urea.

Fe-EDTA protocol

We based our Fe-EDTA protocol on the protocols used for DNA hydrolysis and footprinting [31,43,44]. This protocol was run at 95 °C for 10 min. Screening of several different reaction conditions demonstrated that the direct Fe-EDTA protocol that gave the strongest RNA labeling (Fig. 1C, b) and the strongest hybridization signal was 1.5 mM Fe/3 mM EDTA/10 mM H₂O₂/1 mM NaAsc. Gel electrophoresis demonstrated that this reaction condition produced RNA fragments between approximately 50 and 100 nucleotides in length (Fig. 1D, b). Increasing the concentration of Fe-EDTA 10-fold resulted in an increase in the length of RNA (Fig. 1D, a), a decrease in the RNA labeling (Fig. 1C, a), and a 5-fold decrease in the hybridization signal. This suggests that a higher level of Fe-EDTA inhibited the fragmentation, which resulted in a decrease in the hybridization signal. Increasing the level of H₂O₂ 10 times and increasing the level of Fe-EDTA 100 times resulted in almost complete degradation of the RNA (Fig. 1D, c), a further decrease in the RNA labeling (Fig. 1C, c), and a 20-fold decrease in the hybridization signal compared with optimal conditions. These results indicated that a reduced hybridization signal could occur if the reaction proceeded too far or if the reaction did not proceed far enough. An additional experiment demonstrated the relationship between the hybridization signal and the Fe-EDTA concentration (Fig. 4). In this experiment the H₂O₂ concentration was held at 10 mM, and the Fe-EDTA concentration was varied from 0 to 7.5 mM. The results of this experiment suggest that a low level of Fe-EDTA does not produce an adequate level of fragmentation and labeling, and an excess of

Fe-EDTA inhibits fragmentation and labeling. This experiment also supported 1.5 mM Fe/3 mM EDTA/10 mM H₂O₂ as the optimal reaction conditions for the direct protocol.

An experiment was run to determine if sodium ascorbate is required for the Fe-EDTA reaction. Increasing of NaAsc concentration 10-fold only slightly changed RNA length (compare Fig. 1D, a and d) and labeling (compare Fig. 1C, a and d). Complete removal of sodium ascorbate had no impact on the level of fragmentation (data not shown) and actually resulted in an 2-fold increase in the hybridization signal. Therefore, the Fe-EDTA protocols should be run without NaAsc.

The indirect Fe-EDTA protocol that gave the strongest hybridization signal (1094 u/μg/sec) was 1.5 mM Fe/3 mM EDTA/1 mM H₂O₂. The optimal indirect Fe-EDTA protocol required a 10-fold lower concentration of H₂O₂ than the direct protocol (Table 2). This result was consistent with the results for the OP-Cu system, in which the indirect protocol also required lower reactant concentrations. It was also observed that for the direct Fe-EDTA protocol run at 95 °C the addition of urea did not increase RNA-labeling (data not

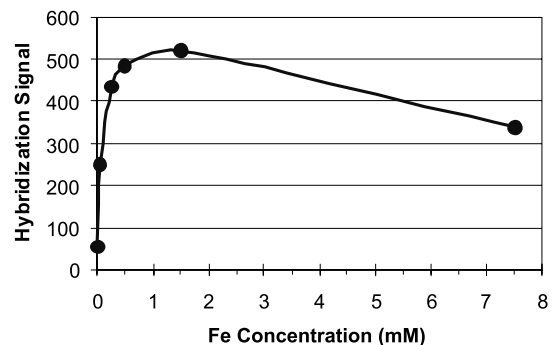


Fig. 4. Relationship between hybridization signal and Fe concentration. Bulk *B. cereus* 9620 RNA fragmented and labeled by direct Fe-EDTA protocol. Fe concentrations used (mM): 0, 0.05, 0.25, 0.5, 1.5, 7.5. All reactions were run with 10 mM H₂O₂ at 95 °C for 10 min.

shown), which was consistent with the results obtained with the OP–Cu protocol.

Comparison of methods

To determine the effectiveness of the OP–Cu and Fe–EDTA systems, we compared the hybridization signals obtained with these systems to the hybridization signal obtained with the magnesium–sodium periodate-labeling and fragmentation method, where RNA was fragmented with a standard magnesium method [1–7,10] and labeled with a standard chemical protocol [11]. The magnesium-sodium periodate method does not modify nucleobases and provides a high yield of 3'-labeled RNA fragments [11]. Both of these features make these fragments highly effective in hybridization with short oligonucleotides (with a length of 10–30 nucleotides) [11], which are commonly used as probes applied on DNA microchips. However, this method is time consuming and it requires a large number of steps (see Materials and methods). We ran direct labeling and indirect labeling variations of all three methods at optimum reagent concentration using *B. cereus* 9620 bulk RNA (Table 2). We found that all three methods gave identical hybridization patterns (Fig. 3) and the hybridization signals for all three methods were approximately equivalent (Table 2). The fact that equivalent hybridization signals were achieved for the radical and non-radical methods indicated that the radicals were not damaging nucleobases in a way that would interfere with hybridization, which agrees with earlier findings [29,50,51].

We found that for both, OP–Cu and Fe–EDTA, systems direct labeling required a 10 times higher concentration of H₂O₂ and, in the case of the OP–Cu protocol, a 10 times higher concentration of coordination complex. This difference between direct and indirect labeling methods may be due to differences in the concentration of the amino groups in reaction, or differences in the efficiency of Schiff base formation for mono- and diamines. The indirect protocol includes 50 mM EDA (diamine) compared to the 1 mM EDA moiety of LissRhod (monoamine) in the direct protocol. The stability of the sulfonyl chloride derivative of TexRed may also be a factor in the difference between the direct and the indirect protocols, since the active sulfonyl group of TexRed has been shown to be much less stable in aqueous solutions than the amino group of LissRhod [45].

We also found that for all three systems, indirect labeling consistently produced 20 to 30% higher hybridization signals than direct labeling. It could be suggested that the lower hybridization signal for direct labeling was due to exposure of LissRhod to radicals in the direct labeling protocols. However, this explanation does not fit the data, since the same difference in direct and

indirect protocols was observed for the magnesium-sodium periodate method, which does not produce radicals. A more likely cause of this consistent difference in signal between direct and indirect protocols is the fact that the quantum yield of TexRed is higher than the quantum yield of LissRhod [45].

Sequence specificity of fragmentation

Sequence specificity of nucleic acid fragmentation is an intrinsic feature of all enzymatic nucleases and results in the appearance of sharp bands on the electrophoresis of the degraded samples [43,44]. We did not find any specific bands on the electrophoresis after labeling-fragmentation of a cRNA represented by single-stranded 300 nucleotide RNA fragments according to the optimized direct OP–Cu and Fe–EDTA protocols either at 95 or at 45 °C (Fig. 5). Bands in the zones “X” and bromophenol blue with fragment length about 40 nucleotides and 20 nucleotides, respectively, represent artifacts of electrophoresis. These bands may be found also on Fig. 1, where bulk bacterial RNA was fractionated. However, when the concentration of the radical-generating components of the reaction were not optimized we sometimes found sequence-specific patterns of fragmentation for the direct OP–Cu protocol performed at 45 °C, but not for the Fe–EDTA protocol (not shown).

Anaerobic conditions

The H-4' abstraction pathway discussed above indicates that amine crosslinking via the radical-mediated protocols may be less effective under aerobic conditions. To achieve anaerobic conditions, we reduced the level of oxygen in all of our reactants and reaction solutions by bubbling with argon (see Materials and methods). To determine if this argon bubbling was necessary, we ran both the OP–Cu and the Fe–EDTA direct protocols with and without argon bubbling using *B. cereus* 9620 bulk RNA. The results indicated that argon bubbling had no impact on fragmentation for either protocol. However, for the OP–Cu protocol, argon bubbling resulted in a 15% increase in hybridization signal. In contrast, for the Fe–EDTA protocol, argon bubbling resulted in a 14% decrease in hybridization signal. Therefore, argon bubbling should be included for the OP–Cu method but not for the Fe–EDTA procedure.

Reduction step

For both the OP–Cu and Fe–EDTA protocols, the reduction step was carried out after the fragmentation and labeling step was completed. An experiment was run for both the OP–Cu and the Fe–EDTA protocols in which the reductant, NaCNBH₃, was included in the

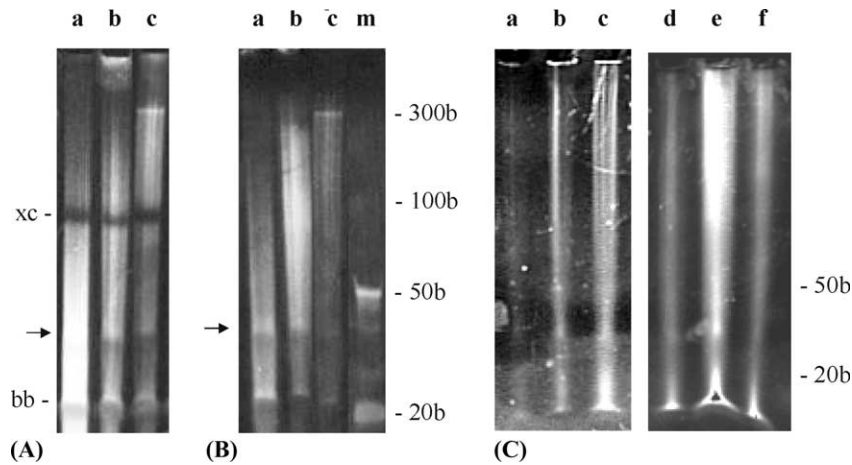


Fig. 5. Labeled RNA and DNA are randomly fragmented. Electrophoresis of single-stranded 300 nucleotide RNA (A,B) after labeling and fragmentation were performed under denaturing conditions in 20% polyacrilamide gel (for details see legend to Fig. 1). (A) Fluorescence image, (B) ethidium bromide-stained gel image. (a, b) direct OP–Cu protocol with 50 mM OP, 5 mM Cu; and 15 mM (a), or 2.5 mM (b) H_2O_2 ; (c) direct Fe–EDTA protocol with 10 mM Fe, 20 mM EDTA, and 10 mM H_2O_2 . Reaction was performed at 95 °C for 10 min (a, c), or at 45 °C for 30 min (b). Arrows indicate position of electrophoresis zone “X” (see text). Fluorescent image of 16S cDNA after electrophoresis in 8% polyacrilamide gel (C). DNA was treated according direct OP–Cu (a–c), or Fe–EDTA (d–f) protocols at 95 °C for 10 min in the presence of 10 mM H_2O_2 and: (a) 7.5 mM OP, 0.75 mM Cu; (b) 15 mM OP, 1.5 mM Cu; (c) 30 mM OP, 3 mM Cu; (d) 0.75 mM Fe, 1.5 mM EDTA; (e) 1.5 mM Fe, 3 mM EDTA; and (f) 3 mM Fe, 6 mM EDTA. Reactions were performed without argon bubbling. OP–Cu labeling was followed by reduction with 20 mM $NaCNBH_3$. The reduction step was eliminated from Fe–EDTA protocol.

reaction with all of the other reagents. For the OP–Cu protocol, the inclusion of the reductant in the reaction step produced a slightly higher level of fragmentation compared to the standard protocol as well as a 1.5-fold increase in hybridization signal. This change in the standard OP–Cu protocol reduced the time required for the protocol by 30 min. For the Fe–EDTA protocol, the inclusion of $NaCNBH_3$ in the reaction step produced a slightly lower level of fragmentation. Also, the inclusion of the reductant resulted in a 3- to 4-fold decrease in the hybridization signal. At the same time, complete exclusion of the reduction step from the Fe–EDTA protocol resulted in no changes in labeling or in fragmentation of bulk bacterial RNA. Therefore, for the Fe–EDTA protocol, the reduction step with $NaCNBH_3$ may be completely excluded, reducing the time required for the protocol by 30 min.

Fragmentation and labeling of DNA

Although this study has mostly focused on the fragmentation and labeling of RNA, DNA can also be used as a target for hybridization with DNA microarrays. We ran both the OP–Cu and the Fe–EDTA protocols using cDNA represented by 16S rDNA, which was produced by PCR amplification of bulk DNA from *B. cereus* T. The OP–Cu protocol was run in the manner described above for both direct and indirect protocols for RNA (Table 2). Both direct and indirect OP–Cu protocols run with DNA resulted in a good hybridization image (Figs. 6A and B) and hybridization signals of 244 and 459 u/ μ g/s,

respectively. The higher hybridization signal for the indirect protocol was consistent with the results for RNA.

We also carried out DNA labeling-fragmentation with versions of the direct OP–Cu and Fe–EDTA protocols optimized for speed. We carried out these reactions at 95 °C for 10 min. without argon bubbling.

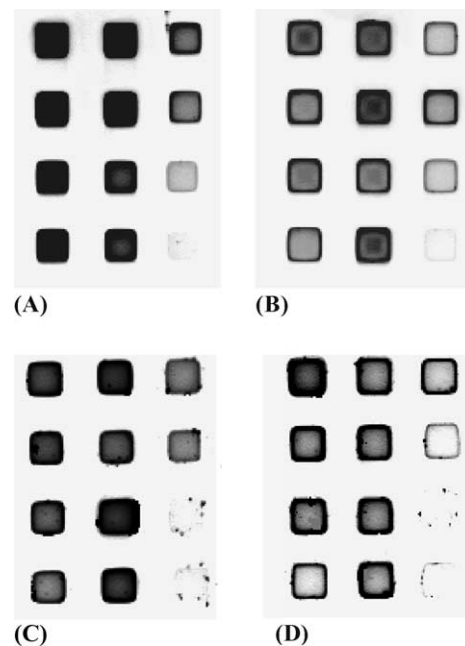


Fig. 6. Hybridization of labeled 16S rDNA produced by PCR amplification of bulk DNA from *B. cereus* T. (A) OP–Cu indirect protocol; (B) OP–Cu direct protocol; (C) Rapid direct OP–Cu protocol; (D) Rapid direct Fe–EDTA protocol.

The reduction step was excluded for the Fe–EDTA procedure. The rapid OP–Cu and Fe–EDTA protocols run with DNA gave good hybridization images (Figs. 6 C and D) and hybridization signals of 119 and 115 $\mu\text{g}/\mu\text{g}/\text{s}$, respectively.

As discussed above, radical-generating systems have been shown to fragment both DNA and RNA [46]. However, due to differences in the structure and composition of DNA and RNA, these molecules may be impacted differently by radical-generating systems. In the current study we have demonstrated that the OP–Cu and Fe–EDTA protocols which were developed for the fragmentation and labeling of RNA may also be used for the fragmentation and labeling of DNA prior to microchip hybridization.

We also tested the sequence specificity of the rapid direct DNA-labeling and fragmentation protocol run at 95 °C. 16S rDNA was labeled at different concentrations of OP–Cu or Fe–EDTA complexes. We did not find any specific bands at the studied conditions (Fig. 5C). These results suggested that DNA, as well as RNA, was not involved in sequence-specific fragmentation at the studied conditions.

Storage of labeled samples

Stability of labeled RNAs in storage has also been examined. We found that samples labeled with the Mg^{2+} -sodium periodate method or the Fe–EDTA method did not decrease their length or their ability to hybridize with oligonucleotides on the microchip after storage at –80 °C for at least 1 year. However, when stored 2–4 months at –80 °C, very seldom OP–Cu-labeled samples were considerably degraded and their capability for hybridization was decreased approximately 5- to 10-fold. This may be due to the continued action of trace levels of the coordination complexes remaining in the samples.

However, precipitation of OP–Cu labeled samples with 3 vol of 96% ethanol containing 0.4 M sodium acetate completely prevented degradation of the nucleic acids. This effect may have been due to ethanol's ability to inhibit radicals.

Which protocol is most convenient?

In most cases, the direct protocol is more simple and quick compared with the indirect protocol. However, some dyes may be unstable at the conditions required for nucleic acid modification (high temperature presence of urea or radicals). In this case, the indirect method may have some advantages. In addition, the direct protocol may be used in the presence of amino-derivative dyes only. The indirect protocol considerably extends the spectrum of the dye derivatives which may be attached to nucleic acid (see above).

Timing optimization

Practical application of a fragmentation and labeling method demands a simplified procedure requiring as little time as possible. As was demonstrated above, the elimination of argon bubbling simplified the procedure without a significant decrease in labeling and fragmentation. This protocol may also be run in a column format that may be done without centrifugation and which should be easily adaptable to automation. Recently, we successfully used the direct OP–Cu protocol without argon bubbling for development of a manually operated universal silica minicolumn for isolation, labeling, fragmentation, and purification of nucleic acids from the excess of dye [37]. The labeling and fragmentation on the column were performed at 95 °C for 10 min using a cocktail containing 5 mM OP, 0.5 mM CuSO_4 , 2 mM H_2O_2 , 20 mM NaCNBH_3 , 1 mM LissRhod, 20 mM sodium phosphate (pH 7.0). This one-column method produced fluorescently labeled nucleic acid samples that were ready for hybridization from human and bacterial cells in 30 min starting from the whole cells. To our knowledge this is the shortest protocol currently available for microarray sample preparation.

The NaCNBH_3 reduction step may also be excluded from the protocol without a considerable decrease of dye incorporation (Fig. 7A). The time required for the labeling-fragmentation reactions may also be considerably decreased by utilizing increased concentrations of OP–Cu and H_2O_2 (Fig. 7B). Using the column format described above [37], we demonstrated discrimination of *B. mycoides* (belongs to the *B. cereus* group) cells (Fig. 7D) from *B. subtilis* cells (Fig. 3G, [37]) with a DNA oligonucleotide microchip employing the OP–Cu protocol without argon bubbling and reduction, where the labeling-fragmentation procedure was performed for 1 min at 95 °C with labeling cocktail containing 15 mM OP, 1.5 mM Cu, 100 mM H_2O_2 , 1 mM LissRhod, and 20 mM sodium phosphate (pH 7.0). Further decreasing the reaction time to less than 1 min is also possible, but this may decrease the reproducibility of the labeling-fragmentation reaction. We hope that automation of the column procedure will remove this restriction. Also, we did not find degradation in samples obtained with a minicolumn procedure when we kept labeled rRNA at –80 °C for 3 months, probably due to effective elimination of the traces of coordination complexes in a thorough washing procedure on the column.

Hybridization specificity of labeled nucleic acids

Previous hybridization experiments done in our lab [37] using *B. thuringiensis*, *B. subtilis*, *E. coli*, and human HL60 cells demonstrated that RNA labeled with the hydroxyl radical system (OP–Cu method) showed a high level of hybridization specificity. In the current work we

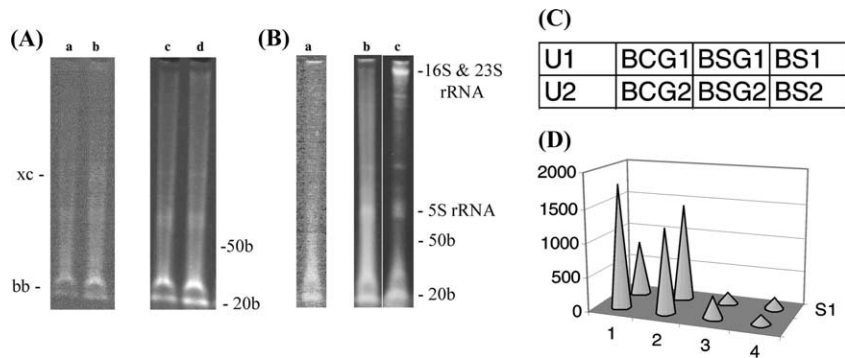


Fig. 7. Column method of sample preparation. *B. mycooides* NCTC8096 (belongs to the *B. cereus* group) bulk nucleic acids were isolated from cells, labeled, fragmented, and purified of the excess dye using a silica minicolumn. (A, B) Electrophoresis of labeled and fragmented bulk nucleic acids under denaturing conditions (for details see legend to Fig. 1). (A) Labeling-fragmentation reaction was carried out at 95 °C 10 min with cocktail containing 5 mM OP, 0.5 mM Cu, 2 mM H₂O₂, and 20 mM sodium phosphate, as described (37), with (b, d) or without (a, c) 20 mM NaCNBH₃ in labeling cocktail. (a, b) Fluorescent image, (c, d) Ethidium bromide-stained gel image. (B) Labeling-fragmentation reaction was performed using a silica minicolumn for 1 min. at 95 °C in labeling cocktail containing 15 mM OP, 1.5 mM Cu, 100 mM H₂O₂, 1 mM LissRhod and 20 mM sodium phosphate, pH 7.0 without reduction (a, b), total nucleic acid before labeling and fragmentation (c). (a) Fluorescent image; (b, c) ethidium bromide-stained gel. (C) Arrangement of probes immobilized on a microchip for identification of "all life" (U1 and U2), *B. cereus* group bacteria (BCG1 and BCG2), *B. subtilis* group bacteria (BSG1 and BSG2), and *B. subtilis* spp. (BS1 and BS2); for details see Table 1. (D) Bulk nucleic acid treated as described in (B) was hybridized for 10 min at room temperature with a microchip (C). Analysis of hybridization image has been done as described under Material and methods with deduction of fluorescence of the empty gel element (i.e., background of both slide and gel were taken into account).

have demonstrated that DNA and RNA fragments labeled with OP–Cu and Fe–EDTA systems, according to both the direct and indirect protocols, were successfully able to differentiate 20 b oligonucleotide probes which are distinct from each other in only two nucleotides (see probes 10 and 11, Table 1, Figs. 2, 3, 6). Recently, we also found that fluorescently labeled RNA fragments obtained using the column OP–Cu method [37], in a hybridization experiment with microchips, may recognize one nucleotide mismatch in 20 b oligonucleotide probes with a perfect:mismatch ratio in the range of 2.5–8.0 (data not shown).

Conclusion

In the present study we have demonstrated the advantages of the radical-generating chemical complexes OP–Cu and Fe–EDTA for labeling and fragmentation both double-stranded and single-stranded nucleic acids against other methods. Both complexes provide labeling and fragmentation simultaneously and effectively, for both RNA and DNA. In accordance with the described chemistry (see above), in the labeling reaction fluorescent dye was incorporated mostly to 3'- or 5'-ends of nucleic acid fragments. End-labeling and the absence of a considerable amount of nucleobase modification, which may disturb hybridization, make these fragments very promising for recognizing and differentiating short sequences containing only one or two mismatches with a DNA microchip bearing short oligonucleotides as probes. Both the OP–Cu and Fe–EDTA reactions may be run at a wide range of temperatures, from 45 to

95 °C, generating sequence nonspecific labeling and fragmentation. The option of running the reactions in the presence of high concentrations of urea, or alternatively, at high temperatures, results in nucleic acid unfolding, which provides a high yield of labeling and fragmentation and eliminates the influence of the nucleic acids 2D structure on the labeling-fragmentation process. This advantage of the method is especially important for the labeling of RNA, which very often has complicated 2D structure.

In the process of treatment according to the developed direct and indirect protocols, active amino or aldehyde groups appeared on the ends of the nucleic acid fragments, providing the possibility for a broad number of fluorescent dye derivatives to be attached to the nucleic acids. Other advantages of these radical-mediated methods are simplicity, low cost, and high speed. Following the direct OP–Cu protocol, it takes only 1 min at 95 °C, to complete the labeling-fragmentation reaction. These advantages, together with high reaction rate, high yield of labeling, high specificity of labeled nucleic acids in hybridization, and application of the procedure into column format, make this method unique and very promising for sample application to DNA microchip technology and automation. The labeling technique, which we have developed should be applicable to any microarray format.

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