# Immobilization Cyclen Copper (II) on Merrifield Resin: Efficient Oxidative Cleavage of Plasmid DNA

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**ABSTRACT:** Merrifield resin-supported cyclen (MRC) was directly prepared by attaching 1, 4, 7, 10-tetraazacyclododecane (cyclen) to Merrifield resin (MR). Subsequent coordination with Cu(II), Co(II), and Ni(II) gave immobilized cyclen complex MRC-Cu, MRC-Co, and MRC-Ni as "solid artificial enzymes." These complexes were characterized by elemental analysis, IR spectroscopy, ICP-AES, and scanning electron microscopy (SEM). Furthermore, the DNA cleavage activities of these complexes were investigated by agarose gel electrophoresis. The results indicated that MRC-Cu was superior to other solid artificial enzymes, and the cleavage process was carried out via oxidative pathway. Moreover, the solid catalyst MRC-Cu was very stable and it could be reused at least four times without any loss of catalytic activity.  J Appl Polym Sci 111: 2485–2492, 2009

**Key words:** cyclen; Merrifield resin; immobilized metal complexes; DNA cleavage; solid catalysis

## INTRODUCTION

Artificial nucleases have been proved to be efficient tools for DNA cleavage, which was intensively studied by chemists and biologists due to their potential application in biotechnology and therapeutic agents.[1–5] In the past few years, many groups reported different systems as artificial nucleases to accelerate the cleavage of DNA.[6–14] So far the transition metal complexes is regarded as one of the most effective synthetic catalysts for the cleavage process. A large number of mononuclear or multinuclear metal complexes have been developed as effective DNA restriction enzymes.[1–3,15–20] 1, 4, 7, 10-tetraazacyclododecane (cyclen) and its derivates as ligands have been widely studied in DNA recognition and restriction because of their strong coordination ability toward a wide range of transition metals.[21–27] In our previous study, several metal compounds containing cyclen unit were synthesized and their catalytic abilities on DNA restriction were also investigated.[28–32] However, to be an ideal artificial biomimetic catalysts, reproduction, large reaction rate, and high selectivity were regarded as important parameters.[33–37] In this regard, the development of immobile artificial enzymes should be a helpful way in this field.

Sortino et al. developed a self-assembled monolayer on the gold surface, which could immobilize double-strand DNA and could induce the cleavage of DNA upon light excitation.[38] Suh and coworkers reported that cyclen metal complex and guanidinium group attached to poly(chloromethylstyrene-*co*-divinylbenzene) could remarkably enhanced its proteolytic activity via hydrolysis of phosphodiester linkage.[33–37] In this study, we reported a kind of solid biomimetic catalyst by immobilization of Cu(II), Co(II), Ni(II) cyclen complex respectively, on Merrifield resin. The results of DNA cleavage experiments indicated that MRC-Cu was superior to other MRC metal complexes, and the cleavage process was carried out without use of any additives.

## EXPERIMENTAL

### Materials and instruments

All chemicals and reagents were obtained commercially and were used without further purification.

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Anhydrous *N*, *N*-dimethylformamide (DMF) was dried and purified under nitrogen by using standard methods and were distilled immediately before use. All aqueous solutions were prepared from deionized or distilled water. Electrophoresis grade agarose and plasmid DNA (pUC19) was purchased from Takara Biotechnology Company, ethidium bromide (EB) was purchased from Sigma. Merrifield's peptide resin (2% crosslinked, 200–400 mesh, 2 meq Cl/g) was purchased from Aldrich. IR spectra were recorded on a Shimadzu FTIR-4200 spectrometer as KBr pellets. Electrophoresis apparatus was a biomeans stack II-electrophoresis system, PPSV-010. Bands were visualized by UV light and photographed using a gel documentation system by the estimation of the intensity of the DNA bands, recorded on an Olympus Grab-IT 2.0 annotating image computer system. Scanning electron microscopy (SEM) was operated on HITECH *S*-450.

### DNA cleavage experiment

pUC 19 DNA (5 μL, 0.018 μg/μL) in $NaH_2PO_4$/$Na_2HPO_4$ (100 mM, pH 7.4) was treated with different MRC-metal complexes, followed by dilution with the $NaH_2PO_4$/$Na_2HPO_4$ buffer to a total volume of 17.5 μL. The samples were then incubated at 37°C for different time, and loaded on a 1% agarose gel containing 1.0 μg/mL EB. A control reaction was carried out using the same conditions as the cleavage reaction but lacking the MRC metal salts. Electrophoresis was carried out at 40 V for 30 min in TAE buffer. Bands were visualized by UV light and photographed followed by the estimation of the intensity of the DNA bands using a Gel Documentation System.

### Preparation of cyclen

Cyclen was prepared according to the literature.[39] To a stirred solution 20 g of trietylenetetraamine was added 34.7 g of *N*,*N*-dimethylformamide dimethyl acetal, then the solution was refluxed for 30 min under nitrogen. The reaction mixture was dried in vacuo to got off-white solid, and recrystallized from THF. The resulting white solid was filtered and isolated in 68% yield (15.1 g). 15.1 g of dry white solid, 23.7 g of 1,2-dibromoethane, and 9.4 g of $K_2CO_3$ were added to a 2-L round-bottom flask which equipped with 700 mL of acetonitrile. The mixture was heated to reflux while being stirred. After 3 h at reflux, filtered to remove $K_2CO_3$ and the filtrate was dried in vacuo to get a pale yellow solid. The yellow solid was dissolved in 10 mL water and heated to 95°C and dropwised the solution of KOH (10 mL, 12 g). Then the mixture was cooled and cyclen precipitated in 45% yield (6.8 g).

### Preparation of MRC and its metal complexes

A total of 700 mg of Merrifield's resin, 172 mg of cyclen, and 150 mg of $K_2CO_3$ were added to 50 mL of anhydrous DMF in a flask, and then the mixture was heated to 80°C for 12 h under $N_2$ atmosphere; the reaction mixture was cooled and filtrated; the residue was washed with DMF, water, and ethanol to give MRC as white solid.

Totally, 200 mg of MRC was added to 30 mL of boiling ethanol, then 200 mg of $Cu(NO_3)_2$ was added, and the temperature was kept for 2 h. The mixture was filtrated and washed with water and ethanol to remove excess copper ion, the product MRC-Cu was then obtained as a light-blue solid. (MRC-Co and MRC-Ni were obtained via same procedure as ocherous and olive solid from $Co(NO_3)_2{\cdot}6H_2O$ and $NiCl_2{\cdot}6H_2O$, respectively).

IR spectra of MR and MRC derivatives were listed as follows:

MR, IR (KBr, $cm^{-1}$): 3023 (ν Ar—CH); 2920, 2850 (ν C—H); 1602, 1492 (ν Ar—C); 1263 (δ C—Cl);

MRC, IR (KBr, $cm^{-1}$): 3023 (ν Ar—CH); 2920, 2799 (ν C—H); 1674 (δ *N*—H); 1600; 1492, (ν Ar—C); 1268 (δ C—Cl).

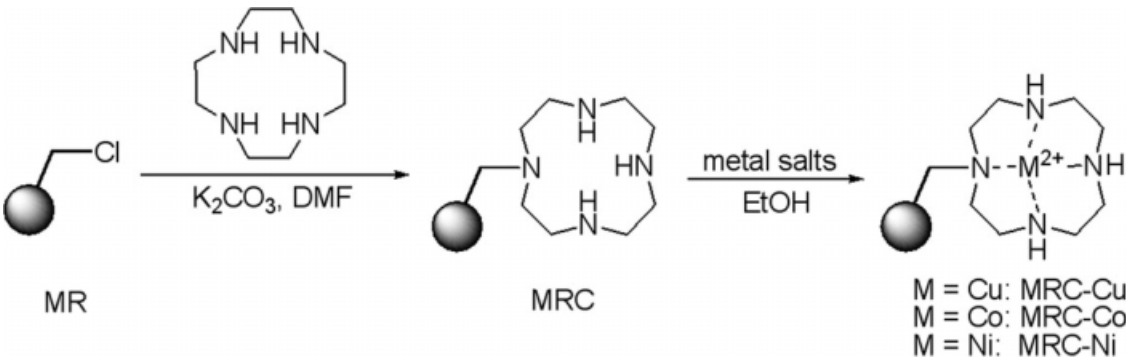
MRC-Cu, IR (KBr, $cm^{-1}$): 3023 (ν Ar—CH); 2921 (ν C—H); 1645 (δ *N*—H); 1600, 1490(ν Ar—C); 1384-1283 (ν skeleton cyclen-Cu).

MRC-Co, IR (KBr, $cm^{-1}$): 3023 (ν Ar—CH); 2920 (ν C—H); 1673 (δ *N*—H); 1601, 1493 (ν Ar—C); 1447-1379 (ν skeleton cyclen-Co).

MRC-Ni, IR (KBr, $cm^{-1}$): 3023 (ν Ar—CH); 2919 (ν C—H); 1672 (δ *N*—H); 1602, 1492, (ν Ar—C); 1378 (ν skeleton cyclen-Ni).

### The DNA inhibition experiment by various scavengers

The DNA inhibition experiment by various scavengers in the presence of MRC-Cu complex has been studied under physiological pH and temperature by gel electrophoresis (1% agarose) using supercoiled pUC 19 DNA as the substrate. In a typical experiment, 2.5 μL of the inhibiting agents (DMSO, 1*M*; *tert*-butyl alcohol, 1*M*; $NaN_3$, 1*M*; SOD enzyme, 2U) were respectively, added to the solution of pUC 19 DNA (5 μL, 0.025 μg/μL) in $NaH_2PO_4$/$Na_2HPO_4$ (100 mM, pH 7.4), followed by dilution with the $NaH_2PO_4$/$Na_2HPO_4$ buffer to a total volume of 17.5 μL. The samples were then incubated at 37°C for 5 h, and loaded on a 1% agarose gel containing 1.0 μg/mL EB. Electrophoresis was carried out at 40 V for 30 min in TAE buffer. Bands were visualized by UV light and photographed followed by the estimation of the intensity of the DNA bands using a Gel Documentation System.




**Scheme 1** Preparation of MRC and relative metal complexes.

## RESULTS AND DISCUSSION

### Preparation and characterization of MRC and its metal complexes

Cyclen could be directly immobilized by the nucleophilic substitution on chloromethyl group of Merrifield resin (Scheme 1). In this reaction, DMF was chosen to be the solvent to swell the resin, and 20 equiv. of cyclen units was used. The reaction mixture was heated for 12 h at 80°C under $N_2$ atmosphere, and after being washed to remove unreacted cyclen, MRC was obtained as white solid. The MRC metal complexes could be prepared from MSC and $Cu(NO_3)_2$, $Co(NO_3)_2$, or $NiCl_2$ in ethanol solution, respectively.

The IR spectra of MR, MRC, and MRC-Cu is illustrated in Figure 1, the peak of chloromethyl in MR is 1263 $cm^{-1}$, after reacted with cyclen, it almost disappeared in MRC; after coordinated with $Cu^{2+}$, the skeleton vibration of the cyclen-$Cu^{2+}$ (broad peak: 1380 $cm^{-1}$) was found.[40,41]

SEM was used to investigate the surface of the MRC, Figure 2 illustrated the shapes of MR and MRC.

Elemental analysis data of MR and MRC were listed in Table I. The percentage of Cl element in Merrifield resin was reduced to <0.3 after being reacted with cyclen, this result indicated that almost all chloride was replaced by cyclen moiety. According to the amount of N% of MRC, the quantity of cyclen in MRC could be easily calculated as 0.81 mmol/g MRC; compared with the %Cl in MR (2 mmol/g), the immobilized rate is 40%. Since there was trace amount of Cl in MRC, one cyclen unit

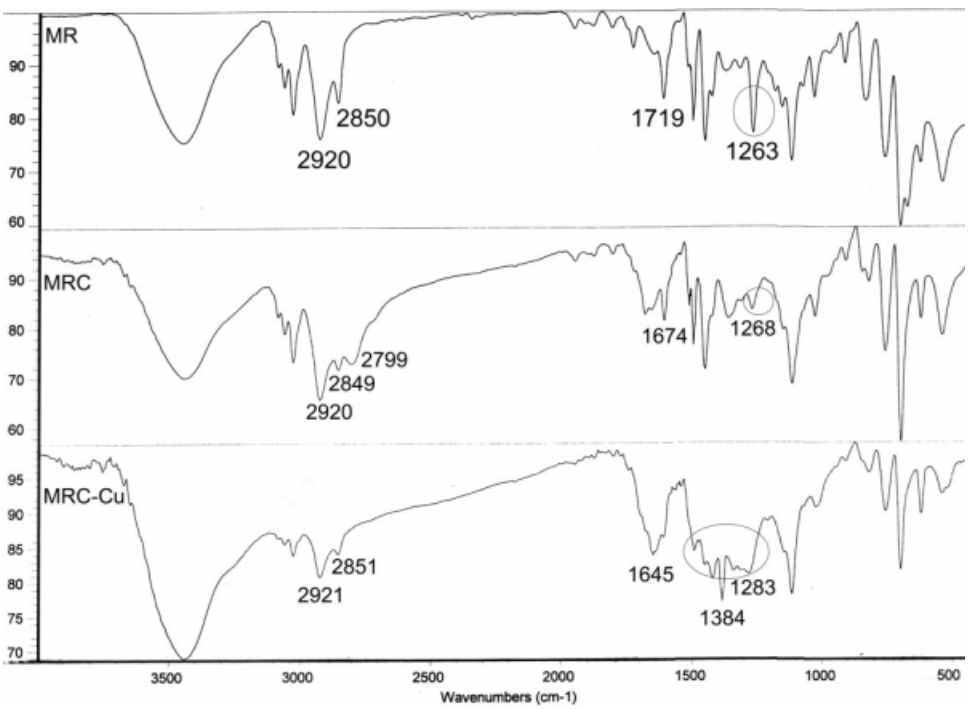



**Figure 1** IR spectra of MR, MRC, and MRC-Cu.

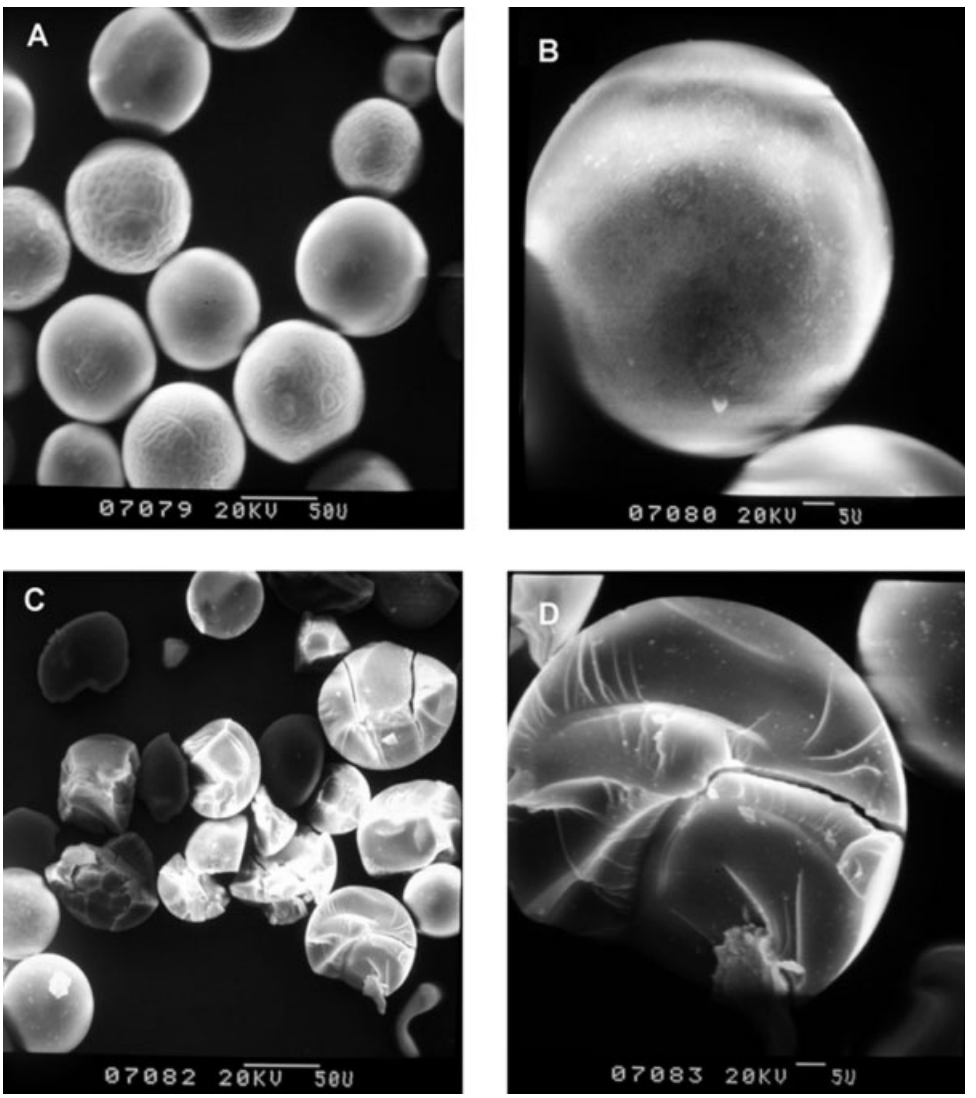



**Figure 2** SEM figures of Merrifield resin (MR, A, B) and Merrifield resin-supported cyclen (MRC, C, D).

may react with two or three equivalent active chloride of MR, which did not influence its coordination with metals.

The amounts of Cu(II), Co(II), and Ni(II) were detected by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The samples were firstly burn in furnace and then the residues were treated with concentrated sulfuric acid to release free metals. The results showed that the immobilized quantity of Cu(II), Co(II), and Ni(II) on MRC was 55.7, 7.8, 20.1 mg/g MRC, respectively, which corresponding 0.87, 0.13, 0.4 mmol/g MRC, based on the cyclen amount of MRC, the complexation radio could be calculated >99% for Cu(II), 16% for Co(II), and 34% for Ni(II). These results indicated that cyclen had stronger coordination to Cu(II) than Co(II) and Ni(II), which is in agreement with their binding ability (lg $K_{Cu(II)} = 23.3$, lg $K_{Co(II)} = 13.8$, and lg $K_{Ni(II)} = 16.4$).[42–45]

### Cleavage of plasmid pUC19 DNA catalyzed by Different MRC-metal complexes

The DNA cleavage activity of MRC metal complexes was then studied under physiological conditions by gel electrophoresis (1% agarose) using supercoiled pUC 19 DNA as substrate. Plasmid pUC19, which contains 2686 bp in length, is a circuler double-stranded DNA. Commonly, it exists as supercoiled form in water solution, which was regarded as

**TABLE I**
**Elemental Analysis of MP and MPC**

| | N (%) | C (%) | H (%) | Cl (%) |
|---|---|---|---|---|
| Cyclen[a] | 32.52 | 55.78 | 11.70 | 0 |
| MR | | 82.72 | 7.05 | 9.14 |
| MRC | 4.54 | 80.54 | 8.26 | <0.3 |

[a] Calculated content.

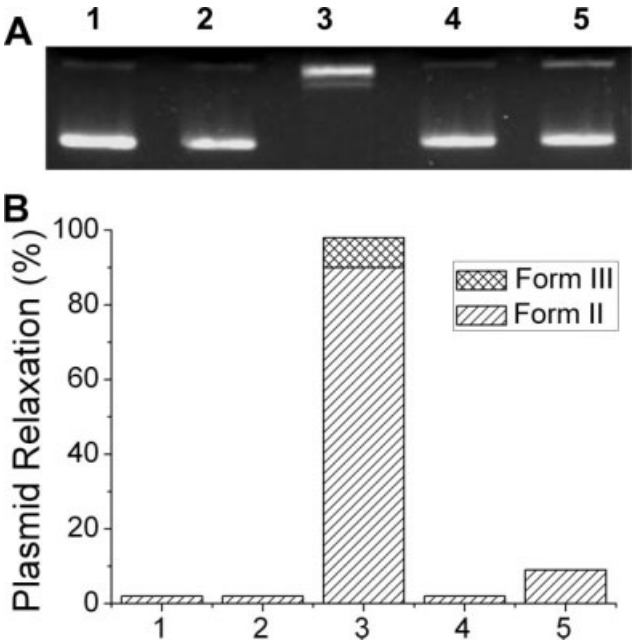



**Figure 3** (A) Agarose gel electrophoresis patterns for the cleavage pUC19 plasmid DNA (5 μg/mL) catalyzed by MRC-metal complexes. Lane 1, DNA control; Lane 2, DNA + Cu-cyclen* (1 mM, 2 μL); Lane 3–5, DNA + MRC-Cu, MRC-Co, MRC-Ni (1.0 mg), respectively. (B) Quantity of % plasmid relaxation (Form II or Form III) relative to plasmid DNA per lane. All reactions were done in a $NaH_2PO_4/Na_2HPO_4$ buffer (100 mM, pH 7.4) at 37°C for 6 h. *Here Cu(II)-cyclen complex was used as control.

"Form I"; when nuclease or artificial nuclease attacked the pUC19, it could cut supercoiled form to be nicked form (or open circular form, relaxed circular form), which was regarded as "Form II"; further attacks could cut the nicked DNA to be linear form, which was regarded as "Form III." The three different form of DNA showed different migration speed in gel electrophoresis; therefore, we can characterized them using EB to stain.[46] Figure 3 showed the results of DNA cleavage catalyzed by these complexes. Among the MRC-metal complexes, MRC-Cu showed excellent catalytic ability, and the conversion of supercoiled plasmid DNA (Form I) was much higher than the reactions catalyzed by MRC-Co and MRC-Ni. MRC-Cu could cleave Form I into Form II and Form III within 6 h, however, MRC-Ni and MRC-Co exhibited poor activity; only small amount of Form II was found in the same time. The reason might be that $Cu^{2+}$ was easier to bind molecular oxygen than $Co^{2+}$ and $Ni^{2+}$ in water,[13,47,48] and the strong coordinate abilities of $Cu^{2+}$ toward cyclen and the higher quantity of copper immobilized on support might also play a role in the cleavage experiment.[33] In the cleavage reaction catalyzed by MRC-Cu, most of the supercoiled plasmid DNA (Form I) was transformed to open-circular form (Form II), and small amount of linear form (Form III) was also detected. Therefore, our subsequent efforts focused on the catalytic ability of MRC-Cu.

### The effects of complex amount and reaction time on DNA cleavage catalyzed by MRC-Cu

The conditions of DNA cleavage reaction catalyzed by MRC-Cu were subsequently optimized. The effect of the amount of MRC-Cu on cleavage activity in 5 h at 37°C was investigated and the results were shown in Figure 4. The conversion of supercoiled plasmid DNA (Form I) increased associated with the increase of MRC-Cu. While the amount was raised to 1.2 mg or more, a large part of Form I was cleaved to fragment, which could not be detected by agarose gel electrophoresis.

Figure 5 displayed the effect of reaction time on the DNA cleavage catalyzed by MRC-Cu. It was clear that the longer of the reaction time, the more amount of nicked DNA (Form II) was obtained. More than 90% yield of Form II was achieved when the reaction was processed for 14 h. The relative amounts of Form I and Form II were quantified by electrophoresis, as shown in Figure 4C, the catalytic cleavage reaction displayed an approximately

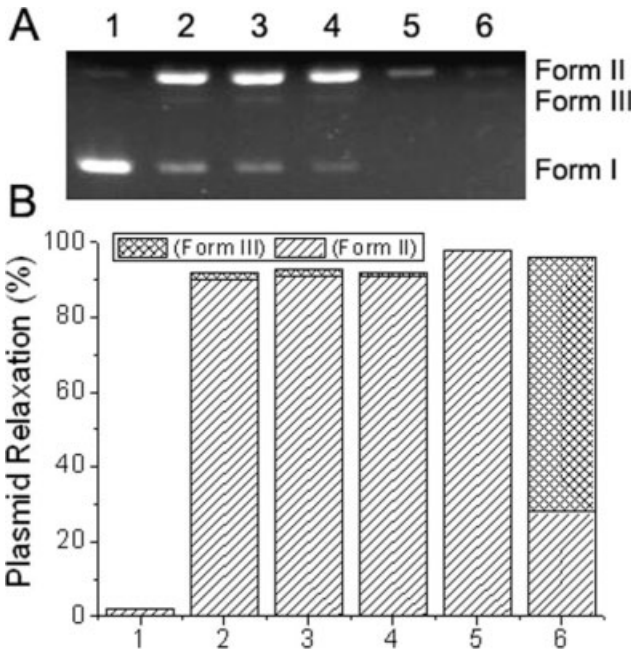



**Figure 4** Agarose gel electrophoresis patterns for the cleavage pUC19 plasmid DNA (5 μg/mL) catalyzed by various weight of MRC-Cu. Reaction was done in a $NaH_2PO_4/Na_2HPO_4$ buffer (100 mM, pH 7.4) at 37°C for 5 h. (A) Lane 1, DNA control; Lane 2–6, DNA + 0.3, 0.5, 0.8, 1.2, 1.8 mg of MRC-Cu. (B) Quantity of % plasmid relaxation (Form II or Form III) relative to plasmid DNA per lane (Lane 5 and Lane 6 showed the radio of Form III/Form II, which was calculated independently based on the amount of DNA in each of them).

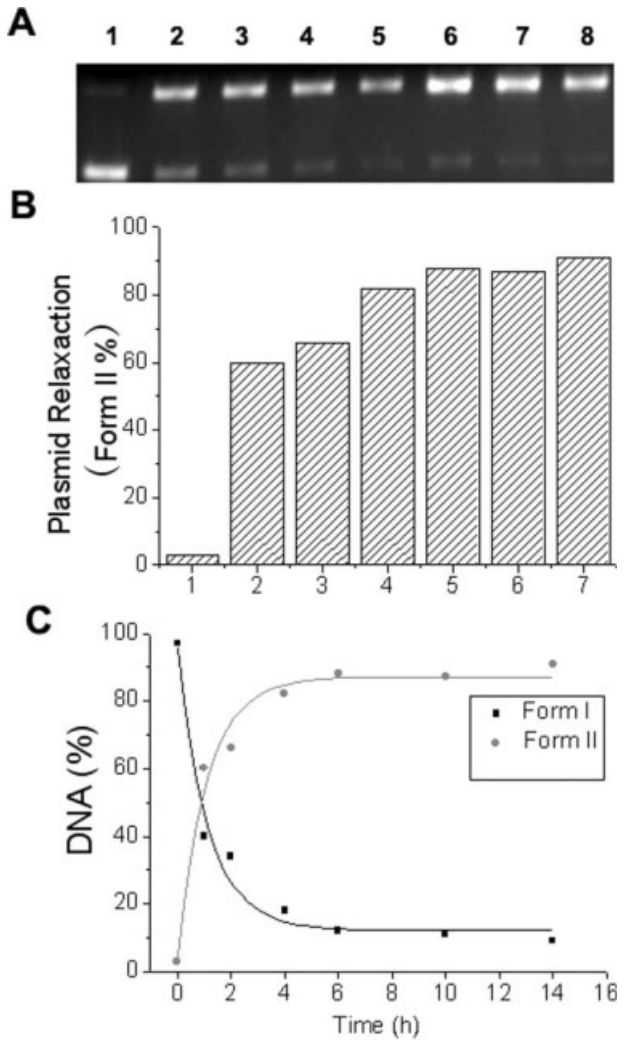



**Figure 5** Time dependence of the cleavage of pUC19 DNA (5 μg/mL) catalyzed by MRC-Cu (0.5 mg) in a $Na_2HPO_4/NaH_2PO_4$ buffer (100 mM, pH 7.4) at 37°C. (A) Agarose gel electrophoresis diagrams of MRC-Cu: Lane 1, DNA control; Lane 2–8, DNA + MRC-Cu, reaction time = 1, 2, 4, 6, 10, 14 h. (B) Quantity of % plasmid relaxation (Form II) relative to plasmid DNA per lane. (C) Reaction curve, which indicated a pseudofirst-order kinetic profile.

pseudofirst-order kinetic behavior[3,49,50] (Fig. 4C), with $k = 0.87\ h^{-1}$ and $R^2 = 0.98$.

### Inhibition of DNA cleavage by radical scavengers

It is well known that some oxidative (e.g., hydrogen peroxide, molecular oxygen) or reductive agents (e.g., ascorbic acid, 3-mercaptopropionic acid (MPA)) could accelerate DNA strand scission.[13] It also reported that Cu(II) could generate reactive oxygen species in aqueous solution.[13,14] To confirm the reactive oxygen species formed in the DNA cleavage process, experiments with a variety of radical scavengers were carried out (Fig. 6). In these studies, DMSO and *tert*-butylalcohol were used as scavengers of hydroxyl radical, sodium azide ($NaN_3$) was used as singlet oxygen scavenger, and superoxide dismutase enzyme (SOD) was used as superoxide scavenger.[51–55] The addition of DMSO and *tert*-butyl alcohol to the system were found to have little effect on the DNA cleavage reactions, which indicated that hydroxyl radicals were not the main effective active species in the processes. On the other hand, the DNA cleavage could be dramatically inhibited when $NaN_3$ or SOD was added to the system. Thus we considered that singlet oxygen or a singlet oxygen-like species were the main reactive species in DNA cleavage reaction catalyzed by MRC-Cu.[51,52]

### Stability and reusability of supported metal-cyclen complex

The MRC-Cu complex was stable in aqueous solution, and it could be easily recovered by filtration and subsequent wash with $NaH_2PO_4/Na_2HPO_4$ buffer (100 mM, pH 7.4). The recovered complex was applied to the same cleavage reaction. Figure 7 showed the results, which indicated that the supported complex could be reused at least four times without any loss of catalytic activity (Form II). In these experiments, no free copper ion was detected (the concentration of $Cu^{2+}$ is less-than the limit detection of ICP-AES), which meant that the copper complex was stable and no catalyst loss was found during the cleavage process.

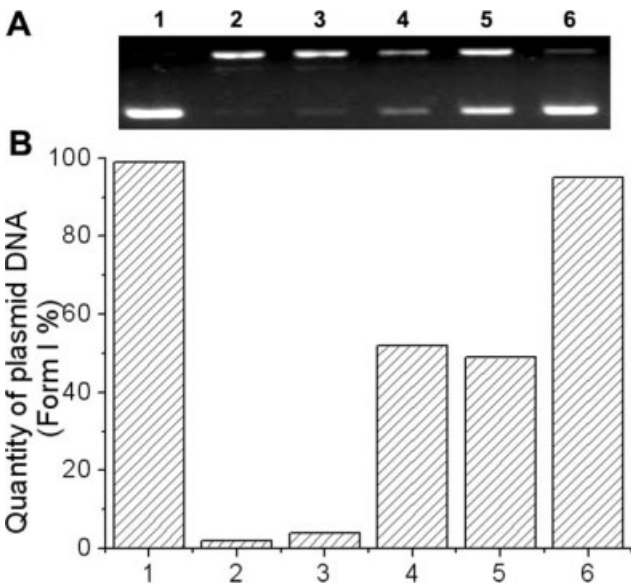



**Figure 6** Inhibition studies on cleavage of pUC19 DNA (5 μg/mL) catalyzed by MRC-Cu, reactions were carried out for 5 h as described in experimental part. (A) Lane 1, DNA control; Lane 2, DNA +MRC-Cu (0.8 mg); Lanes 3–6: DNA + MRC-Cu (0.8 mg) + 143 mM of *tert*-butyl alcohol, DMSO, SOD, $NaN_3$; (B) Quantity of % plasmid DNA (Form I) relative to per lane.

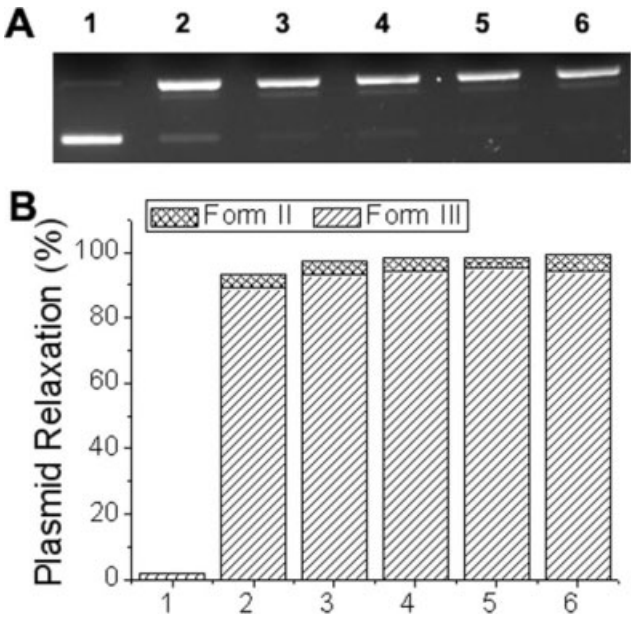



**Figure 7** (A) Agarose gel electrophoresis patterns for the cleavage of pUC19 plasmid DNA (5 μg/mL) catalyzed by reused MRC-Cu catalyst; (B) quantity of % plasmid relaxation (Form II and Form III) relative to plasmid DNA per lane. Reaction was done in a $NaH_2PO_4$/ $Na_2HPO_4$ buffer (100 mM, pH 7.4) at 37°C for 5 h. (A) Lane 1, DNA control; Lane 2, DNA + MRC-Cu; Lanes 3–6, DNA + recovered MRC-Cu (0.8 mg) for run 1, 2, 3, and 4.

## CONCLUSIONS

In summary, we successfully immobilized cyclen Cu(II), Co(II), Ni(II) complex to Merrifield resin, and the catalytic DNA cleavage activity of the result complexes were studied. The results demonstrated that MRC-Cu was an excellent heterogeneous artificial chemical nucleases, it could catalyze the cleavage of plasmid DNA via an oxidative pathway with high conversion and selectivity. Kinetic studies revealed that the DNA restriction procedure followed pseudofirst-order kinetic behavior with $k = 0.87\ h^{-1}$. The MRC-Cu catalyst was stable and easy to be recovered, and it could be reused at least four times without any loss of catalytic activity. Further studies and corresponding experiments are underway to open out the detail mechanism.

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