COLORATIE ADN CU NANOPARTICULE DE METAL PRIN AMINAZA DE REDUCTIE

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ABSTRACT: Interesul crescut in ceea ce priveste culoratia si ordonarea ADN necesita dezvoltarea unor teste suplimentare cu nanoparticule, ca niste markeri alternativi ai vopselelor fluorescente, standard. Acest raport descrie abordarea chimica pentru imbinarea selectiva a particulelor de aur pe scara nanometrica, cu grup de zahar din purine de azot care contin oligonucleotide. Pentru a realiza o singura rasucire a oligonucleotidelor, mai intai trebuie indepartata baza nucleica de pe coloana, prin rasina cu schimb de ioni. Dupa ce nanoparticulele de aur devin amino-functionale si sunt anexate in mod covalent, prin reductia de amine la grupul functional al aldehidelor din lantul deschis al formei de deoxiriboza. Acidul nucleic, modificat cu nanoparticule de aur este stabil in solutii pe baza de apa si acest lucru poate fi observat prin transmisia unui microscop pe electron. Datorita faptului ca particulele individuale, microscopice pot fi detectate usor, aceasta tehnica de etichetare deschide drumul pentru caracterizarea microscopica, pe baza de electroni, a ADN-ului cu o rezolutie ridicata pe scara nanometrica.

CUVINTE CHEIE: Marcaj ADN, particule de aur pe scara nanometrica, aminaza de reductie

1. INTRODUCERE

Interesul in vizualizarea ADN-ului prin microscopie elctronica (EM) a crescut impreuna cu dezvaluirea prin EM a legaturii intre ultrastructura si continut genetic. Nanoparticulele de aur (NPs) par sa fie aproape la fel de bune ca atomii grei pentru etichetarea diverselor tipuri de substante biologice. Acestia prezinta un mijloc de atomi grei care permit localizarea unor spatii specifice prin microscopie electronica, fie direct sau dupa o forme de procesare a imaginii. Nucleul este invelit intr-o carcasa de liganzi organici, facand posibila prinderea fasciculului covalent, de substrat (Jahn, 1999). NPs sunt stabile in solutii amortizoare cu un pH fiziologic si nu sunt

DNA STAINING WITH METAL NANOPARTICLES BY REDUCTIVE AMINATION

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ABSTRACT: The growing interest in DNA staining and sequencing necessitate to develop complementary nanoparticle probes as alternative markers of the standard fluorescence dyes. This report describes chemical approach for selectively conjugation of nanometer-scale gold particles with the sugar moiety of purine nitrogen-containing oligonucleotides. To perform single stranded oligonucleotide labeling, first the nucleobase must be removed from the backbone by ion-exchange resin. After that amino-functionalized gold nanoparticles are covalently attached by reductive amination to the aldehyde functional group of the open chain deoxyribose form. The modified nucleic acid with gold nanoparticles is stable in aqueous solution and it can be observed by transmission electron microscope. Because individual label particles can be easily detected, this labeling technique opens the way for electron microscopic characterization of DNA with high resolution in the nanometer scale.

KEY WORDS: DNA-labeling, Nanometer-scale gold particles, reductive amination.

1. INTRODUCTION

Interest in techniques for visualizing DNA by electron microscopy (EM) has increased greatly with the impact of EM to reveal the correlation between ultrastructure and genetic content. Gold nanoparticles (NPs) appear to be almost ideal as heavy atom labels for labeling of various types of biological substances. They display a core of heavy atoms, which allows the localization of specific sites by electron microscopy either directly or after some form of image processing. The core is surrounded by a shell of organic ligands, making it possible to attach the cluster covalently to the substrate (Jahn, 1999).

foarte sensibile la oxigen. Etichetarea citochimica cu ajutorul particulelor de aur a devenit populara de cand markerul este electrodens si cuantificabil. Un DNA cu dubla rasucire, dotat cu nanoparticule de aur poate di observat cu usurinta, prin transmisie cu ajutorul microscopiei de electroni (TEM) (Hiriyanna *et al.,* 1988).

Strategia pentru cuplarea covalenta a nanoparticulelor la materialul biologic este un pas important in tehnologia de etichetare cu ajutorul biofocalizarii. O posibilitate este prinderea nanoparticulelor de amine, modificate cu ajutorul bazei Schiff, urmata de o reductie moderata a legaturii C-N (Lipka *et al.*, 1983). Bazele Schiff, formate printr-un grup de amine pot fi reduse cu ajutorul unui tratamentului cu cianoborohidrura de sodiu pentru a oferi o legatura covalenta, stabila (Hainfeld and Furuya, 1995). Aceasta reactie este cunoscuta ca o aminaza de reductie. Aminaza de reductie, acolo unde un amestec al unei aldehide cu o cetona sau amina este tratat cu un reducator intr-o forma singulara, este una dintre cele mai utile si versatile metode de pregatire a aminelor si a compusilor functionali din sistemele chimice si biologice (Robichaud and Ajjou, 2006). Pe de alta parte, in cazul acizilor nucleici, este posibila realizarea hidrolizei legaturii glicozidice- *N1* intre bazele de azot si jumatate de deoxiriboza in cadrul conditiilor acide. Bazele purinice sunt hidroloze mai rapide decat pirimidinele. Aceasta reactie ofera grupuri de aldehide reactive, in locul bazelor de nuclei Adenosine sau Guanine in lantul oligonucleotidic. Grupurile de aldehide pot fi etichetate cu succes cu ajutorul nanoparticulelor de amine, modificate prin aminaza de reductie. Scopul acestei lucrari este de a verifica toate conditiile selective pentru hidroliza bazelor de purine si etichetarea continutului de zahar cu nanoparticulele de aur.

NPs are stable in buffer solution at physiological pH and are not very sensitive to oxygen. Cytochemical labeling with gold nanoparticles is becoming popular since the marker is electrondense and quantifiable. Single or double-stranded DNA decorated with gold NPs can be easily imaged by transmission electron microscopy (TEM) (Hiriyanna *et al*., 1988).

The strategy for covalent binding of nanoparticles to the biological material is a crucial step in bioimaging labeling technology. One possibility is the attachment of amino-modified nanoparticles via a Schiff base, followed by mild reduction of the C-N bond (Lipka *et al.*, 1983). The Schiff bases formed with amino group can be reduced by treatment with sodium cyanoborohydride to give stable covalent bond (Hainfeld and Furuya, 1995). This reaction is well known as reductive amination.

Reductive amination, where mixture of an aldehyde or ketone and amine is treated with a reductant in one-pot fashion, is one of the most useful and versatile methods for preparation of amines and related functional compounds in chemical and biological systems (Robichaud and Ajjou, 2006). On the other hand, in the case of nucleic acids, it is possible to hydrolyse the *N1*-glycosidic bond between nitrogen bases and deoxyribose moiety under acidic conditions.

The purine bases are hydrolyses much more rapidly than the pyrimidines. This reaction provides reactive aldehyde groups instead Adenosine or Guanine nucleobase in the oligonucleotide chain. The aldehyde groups can be labeled successful with amino-modified nanoparticles by reductive amination. The purpose of this report is to investigate selectively conditions for hydrolysis of purine bases and to label their sugar moiety with gold nanoparticles.

2. DECTIUNE EXPERIMENTALA

Etichetare deoxiriboza cu ajutorul aminazei de reductie. O solutie 2-Deoxiriboza (100 μL solutie def DMF cu 0.05 M deoxiriboza si 3M CH_3COOH) a fost adaugata la 100 μL 0.5 M solutie de pirenmetilamina si incubata 30 min. Culoarea amestecului s-a modificat de la galben la portocaliu. Dupa perioada de incubatie, a fost adaugat un volum echivalent cu agent reducator de 0.5 M ($NaCNBH_3$, HCOOH, $(Et)_2SiH_2$) in DMF la amestecul reactiei. Monitorizarea reactiei a fost realizata prin intermediul unei analize TLC (solvent CH_3OH : CH_2Cl_2 in procent 3:1) si test de ninhidrina pentru a vizualiza cantitatea finala de produs. Procentul reactiei a fost catalizat in prezenta unui aditiv (2-piridina, TFA, $[RhCp^*Cl_2]_2$, $[Ir(cod)Cl]_2$) asa cum a fost mentionat in Tabelul 1, 2.

Eliminarea purinei unui grup de 2'-grup de depurinizare si etichetare a oligonucleotidelor cu nanoparticule de aur. Intr-un recipient cu baza rotunda, de 20 ml, se adauga graduator dAC deoxiribonucleozida (0.079 mmol, Fig. 4), 2.7 ml MilliQ H_2O si 100 eq, Dowex-50WX8-200 rasina schimb ioni (2.2 g, $-SO_3H$ grupuri, 100 eq, 27 ml). Dizolvarea dA este foarte lenta. Monitorizarea reactiei este realizata prin intermediul analizei TLC. Aceeasi abordare este utilizata pentru depurinizarea adenine si a guanineisi pentru etichetarea lantului principal (lungime nucleotide dA_{25}-dT_{25}-dG_{25}-dC_{25} 100 mer) cu nanoparticule de aur, amino-modificate. Oligonucleotidele etichetate au fost observate prin transmisiune microscopica a electronului de 100 kV (JEOL).

3. REZULTATE SI DISCUTII

Strategia noastra de etichetare a oligonucleotidelor este bazata pe depurinizarea bazelor purinice (Adenina) intr-o stare acida, specifica, si conform anexarii nanoparticulelor amino-modificate la legatura glicozidica a deoxiribozei prin aminaza de reductie. Pentru a studia, cea mai buna situatie pentru reactia de etichetare a 2-deoxiribozei a fost utilizata ca un

2. EXPERIMENTAL SECTION

Labeling of deoxyribose by reductive amination. 2-Deoxyribose (100 μL solution of DMF with 0.05 M deoxyribose and 3M CH_3COOH) was added to 100 μL 0.5 M solution of pyrenemethylamine and incubated 30 min. The mixture color was changed from yellow to orange. After incubation time was added equivalent volume 0.5 M reductant ($NaCNBH_3$, HCOOH, $(Et)_2SiH_2$) in DMF to the reaction mixture. The reaction monitoring was done with TLC analysis (solvent CH_3OH : CH_2Cl_2 in ratio 3:1) and ninhydrin test to visualize the amount of end product. The reaction rate was catalyzed in the presence of additive (2-pyridone, TFA, $[RhCp^*Cl_2]_2$, $[Ir(cod)Cl]_2$) as it is shown on Table 1, 2.

Depurination of 2'-deoxyriboadenosine moiety and labeling of oligonucleotide with gold nanoparticles. In 20 ml round bottom flask are added dAC deoxyribonucleoside dimmer (0.079 mmol, Fig. 4), 2.7 ml MilliQ H_2O and 100 eq, Dowex-50WX8-200 ion-exchange resin (2.2 g, $-SO_3H$ groups, 100 eq, 27 ml). The dissolving of dA is very slow. The reaction monitoring is done with TLC analysis. Same approach is used for depurination of adenine and guanine and labeling of backbone chain (oligonucleotide length dA_{25}-dT_{25}-dG_{25}-dC_{25} 100 mer) with amino-modified gold nanoparticles. The labeled oligonucleotides were observed by 100 kV transmission electron microscope (JEOL).

3. RESULTS AND DISCUSSION

Our strategy for labeling of oligonucleotide is based on the depurination of purine bases (Adenine) at specific acidic condition and following attachment of amino-modified nanoparticle to the glycosidic bond of deoxyribose by reductive amination. To be studied the best condition for labeling reaction 2-

compus model al grupului de zahar in oligonucleotide. In solutii de apa, deoxiriboza primara este in echilibru ca un amestec a trei structuri: forma liniara H-(C=O)-(CH_2)-($CHOH)_3$-H si doua inele de deoxiribofuranoza, cu un inel format din cinci inele si o deoxiribopiranoza cu inel format din sase piese. Atunci cand grupul de semi-acetal hidroxil este liber sau nu este legat de baza purinica, ca in cazul formei liniare, forma de aldehida este disponibila pentru reactie cu grupul de amine de pe eticheta, asa cum este indicat in Fig 1.

deoxyribose was used as a model compound of sugar moiety in oligonucleotide.

In aqueous solution, deoxyribose primarily exist in equilibrium as a mixture of three structures: the linear form H-(C=O)-(CH_2)-($CHOH)_3$-H and two ring forms deoxyribofuranose, with a five-membered ring, and deoxyribopyranose with a six-membered ring. When the hemi-acetal hydroxyl group is free or not linked to purine base, as in the case of linear form, the aldehyde form is available to react with amino-group from the label as it is shown on Fig 1.

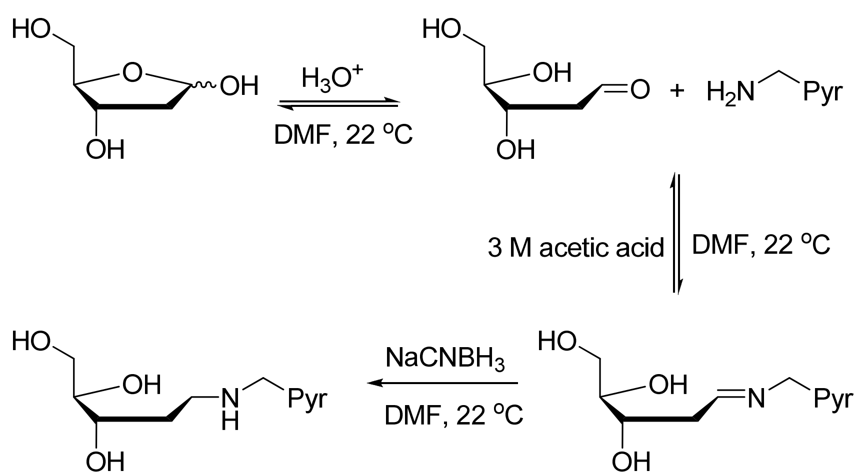


Figura 1. Etichetare 2-deoxiriboza cu pirenmetilamina prin amina de reductie.

Figure 1. Labeling of 2-deoxyribose with pyrenemethylamine by reductive amination.

Aceasta reactie incepe la o temperature a mediului (22 ˚C) in prezenta dimetilformamidei, care ajuta la formarea bazei Schiff ca intermediar. Analiza TLC arata ca rata reactie creste la temperaturi ridicate (72 ˚C). Conditiile specifice ale reactiei si procentul de eficienta al zaharului etichetat, sunt prezentate in Tabelul 1.

This reaction proceeds at ambient temperature (22 ˚C) in the presence of dimethylformamide, which advantages the formation Schiff base as intermediate. TLC analysis demonstrates that reaction rate is increasing at higher temperature (72 ˚C). Specific reaction conditions and yield percentage of the labeled sugar are shown on Table 1

Table 1. Labeling of 2-deoxyribose with pyrenemethylamine by reductive amination at various conditions./ **Tabel 1.** Etichetare deoxiriboza 2 cu pirenmetilamina prin aminare de reductie, la diverse etape.

entry	deoxyribose, mM	label, mM	reductant, mM	additive	t, ºC	yield, %	time, min
1	50	100	$NaCNBH_3$, 500	CH_3COOH	72	> 90	60
2	50	100	HCOOH, 100	$[RhCp^*Cl_2]_2$	72	> 90	30
3	50	100	HCOOH, 100	$[Ir(cod)Cl]_2$	72	> 90	30
4	50	100	$(Et)_2SiH_2$, 100	TFA	22	~ 70	50

De pe eticheta se poate concluziona ca reactia are succes in prezenta acidului acetic in calitate de co-catalizator. Poate fi utilizat in timpul reactiei compusilor cu grup functional, acid, sensibil, cum ar fi acetalul. Anumite metale ca Rh si Ir dar si complexele acestora (Tabelul 1) sunt utilizate pentru hidrogenare ca o cataliza eficienta. In acest caz, procentul reactiei este mai mare. Hidrura de siliciu cum ar fi $(Et)_2SiH_2$ promoveaza reductia efectiva, insa prezenta acidului trifluoracetic poate descompune zaharul la o concentratie si temperatura mai mare. Mutarotatia zaharului si capacitatea de a reactiona cu grupul de amine poate fi imbunatatit, in mod semnificativ, prin adaugarea 2-piridina, ca un catalizator (Capon, 1969) pentru aceasta reactie dependenta de proton (asa cum este indicat in Fig. 2).

From the table it might be conclude that reaction proceeds successfully in the presence of acetic acid as co-catalyst. It can be used during reduction of compounds with acid sensitive functional group such as acetals. Some metals as Rh and Ir and their complexes (Table 1) are used for hydrogenation as effective catalysis. In this case the reaction rate is higher. Silicon hydrides such as $(Et)_2SiH_2$ promote effective reduction, but however the presence of trifluoroacetic acid might decompose the sugar at higher concentration and temperature. The mutarotation of sugar and the ability to react with amino-group can be improved significantly by adding of 2-pyridone as catalyst (Capon, 1969) of this proton-dependent reaction (as it is shown on Fig. 2).

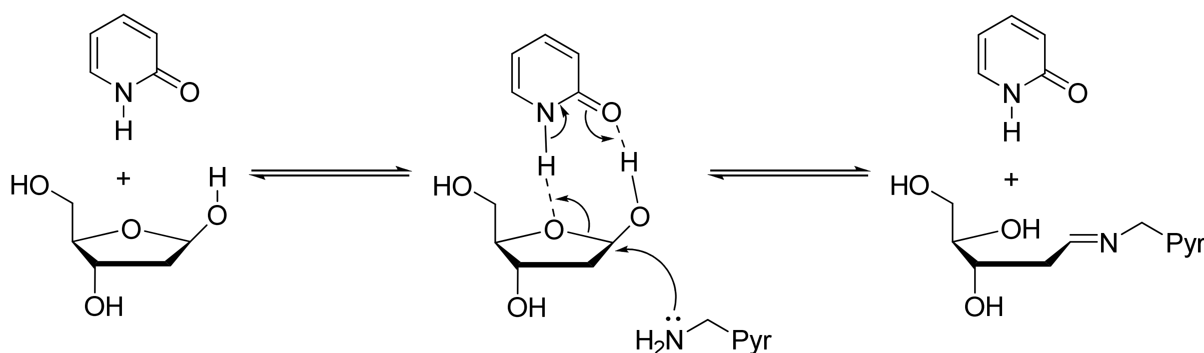


Figura 2. Mecanismul de reactive, propus pentru deschidere inel cu catalizator 2-piridina.

Figure 2. Proposed reaction mechanism of ring opening by 2-pyridone catalyst.

In experimentul real, etichetarea graduatorului de deoxiribonucleozide (inclusiv purina si pirimidina) cu pirenemetilamina, poate fi realizata in loc de 2-deoxiriboza. Conditiile reactiei (a se vedea Tabel 2) sunt blande si eficiente, si se bazeaza pe experimentul de mai sus, cu 2-deoxiriboza. Exista 16 posibile combinatii ale graduatorului de nucleotide, asa cum este indicat in Fig. 3. Insa, experimentele au fost realizate doar cu graduator de deoxiribonucleozida Dac.

In the real experiment the labeling of deoxyribonucleoiside dimmer (including purine and pyrimidine) with pyrenemethylamine is done instead of the 2-deoxyribose. The applied reaction conditions (see Table 2) are mild and effective, and they are based on the above experiment with 2-deoxyribose. There are 16 possible combinations of nucleotide dimmers as they are shown on Fig. 3. However, the experiments were performed only with dAC deoxyribonucleoside dimmer.

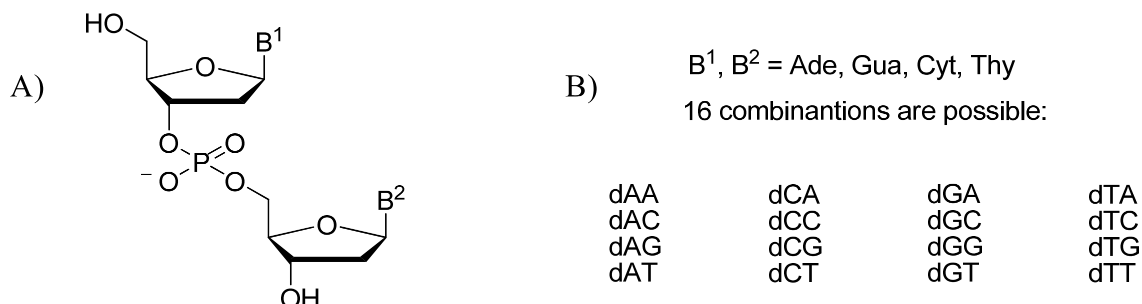


Figura 3. (A) Graduatori de deoxiribonucleozide (B) 16 combinatii posibile intre dA, dG, dC si dT.

Figure 3. (A) Deoxyribonucleoside dimmers and (B) 16 possible combinations between dA, dG, dC and dT.

Un punct important pentru acest experiment este testarea conditiilor de etichetare pe graduator, care contine atat baza purinica sau si pirimidina. Aceste nucleotide pot fi dAC. Dupa cum este indicat in Fig. 4, in cadrul tratamentului cu acid Dowex-50WX8-200 rasina schimbului de ioni a bazei de adenine se poate pierde (*i.e.*, depurinizare). Grupul de amine al etichetei participa in reactiea aminazei de reductie avand ca rezultat spatial abazic. Produsul obtinut poate fi demonstrat fara o viitoare purificare prin analiza TLC sau MALDI-MS.

An important point for this experiment is to test labeling conditions on dimmer, which contains both purine and pyrimidine bases. Such appropriate dinucleotide could be dAC. As it is shown on Fig. 4, under acid treatmen with Dowex-50WX8-200 ion-exchange resin the adenine nucleobase could be lost (*i.e.*, depurination). Then amino group of the label participate in reaction of reductive amination with resulting abasic site. The obtained product can be proved without future purification by TLC or MALDI-MS analysis.

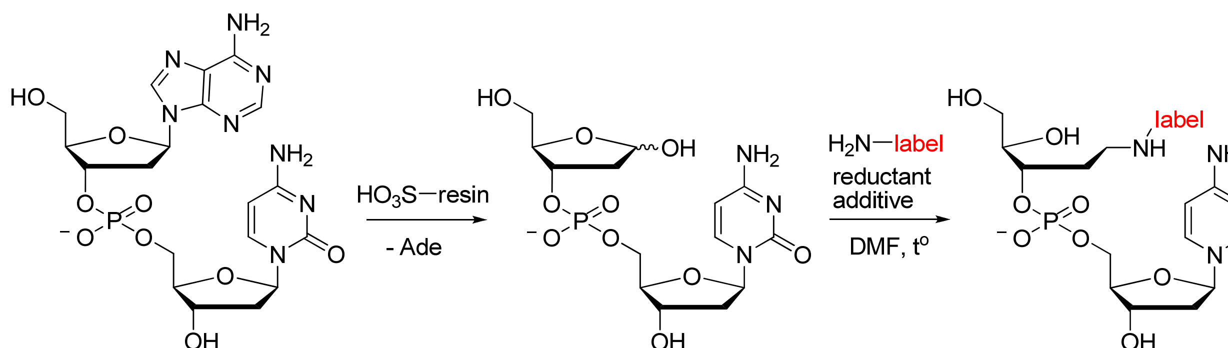
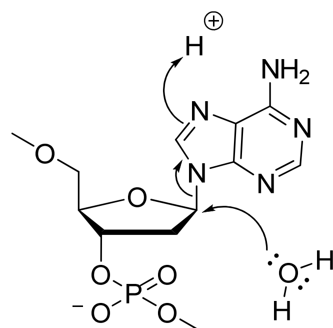


Figure 4. Depurinizare a graduatorului de oligonucleotide si etichetarea spatiului fara baza prin aminare de reductie.

Figure 4. Depurination of dimmer oligonucleotide and labeling of the abasic site by reductive amination.

Mecanismul depurinizarii induse de acid este indicat in Fig. 5. Adenozina este mai sensibila din citozina in aceste conditii acide. Poate fi supusa hidrolizei si grupul de zaharuri care rezulta poate fi etichetat in mod specific, cu particule nanotest. O astfel de abordare detine aplicatii potentiale pentru dezvoltarea unor noi generatii de nanoteste prin ordonarea ADN.

The mechanism of acid-induced depurination is shown on Fig. 5. Adenosine is more sensitive from cytosine at these acid conditions. It can be easily hydrolyze and the resulted sugar moiety can be labeled specifically with nanoprobe particles. Such approach possesses potential application for development of new generation nanoprobes for DNA sequencing.



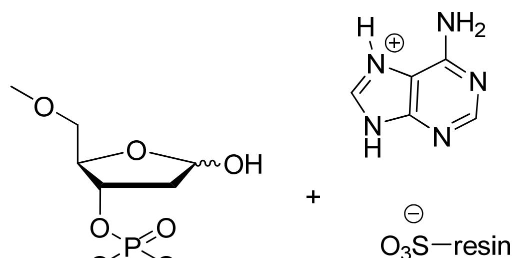


Figura 5. Mecanism de reactive pentru depurinizarea adeninei.

Figure 5. Reaction mechanism of adenine depurination.

Informatiile noastre indica fapul ca in cazul prezentei graduatorului de dinucleotide din acidul acetic (pH ~ 4) este necesara realizarea reactiei de etichetare prin aminaza. In cazul in care acidul acetic se modifica prin catalizatorul de metal, eficienta este scazuta. Totusi, reactia este mai putin eficienta in cazul in care hidrura de siliciu este utilizata (*a se vedea* Tabel 2). Aceste experimente sunt realizate la fel ca 2-deoxiriboza. Insa, prezenta grupului de fosfati si nucleobaza reflecta asupra valorilor obtinute.

Our data indicate that in the case of dinucleotide dimmer the presence of acetic acid (pH ~ 4) is strongly required to complete reaction of labeling by reductive amintaion. If the acetic acid is exchange with metal catalyst the efficiency is lower. Nevertheless, the reaction is with lower efficient if silicon hydrides are used (*see* Table 2). These experiments are performed as same as 2-deoxyrbose. However, the presence of phosphate group and nucleobase reflects on obtained values.

Table 2. Labeling of dAC dimmer oligonucleotide with pyrenemethylamine by reductive amination at various conditions./ Tabel 2. Etichetarea graduatorilor de oligonucleotide dAC cu pirenmetilamina prin aminaza de reductive, in diverse situatii.

entry	dinucleotide, M	label, M	reductant, mM	additive	t, ºC	yield, %	time,min
1	0.0005	0.005	$NaCNBH_3$, 500	CH_3COOH	72	> 90	60
2	0.0005	0.005	HCOOH, 100	$[RhCp^*Cl_2]_2$	72	> 60	30
3	0.0005	0.005	HCOOH, 100	$[Ir(cod)Cl]_2$	72	> 60	30
4	0.0005	0.005	$(Et)_2SiH_2$, 100	TFA	22	~ 20	50

In experimental final, oligonucleotidele include toate cele patru baze de nucleu (dA_{25}-dT_{25}-dG_{25}-dC_{25}) si lungimea de 100 mer este testata pentru etichetare cu nanoparticule de aur prin applicare conditiilor de reactie in intrarea 1, Tabel 2 pentru aminaza de reductie. Schema de reactive este prezentata in Fig. 6.

In the final experiment oligonucleotide including all four nucleobase (dA_{25}-dT_{25}-dG_{25}-dC_{25}) and length of 100 mer is tested for labeling with gold nanoparticles by applying reaction conditions in entry 1, Table 2 for reductive amination. The reaction scheme is shown on Fig. 6.

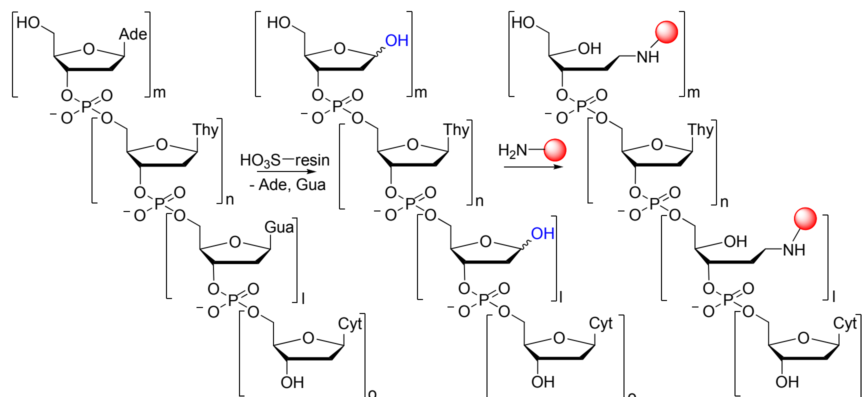


Figura 6. Colorarea oligonucleotidelor cu nanoparticule de aur, amino-modificate prin aminaza de reductie.

Figure 6. Staining of oligonucleotide with amino-modified gold nanoparticles by reductive amination.

ADN-ul colorat apare in sectorul clar al TEM ca un lant de nanoparticule, asa cum este indicat in micrografia de mai jos (*a se vedea* Fig. 7). Probabil, mostra este prea concentrate datorita faptului ca este mai usor sa faci diferenta intre lanturi de oligonucleotide, simple. Insa, aceasta analiza prezinta faptul ca nanoparticulele sunt aranjate, ceea ce se datoareaza legaturii covalente cu coloana de molecule.

The stained DNA appears in the bright-field TEM as chain of nanoparticles as it is shown on the micrograph below (*see* Fig. 7). Probably the sample is too concentrated because it is difficult to distinguish singles oligonucleotide chains. However, this analysis shows that the nanoparticles are arrangement, which is due to the covalent bond with the molecule backbone.

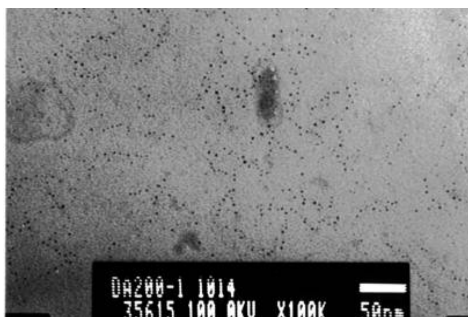


Figura 7. Transmiterea imaginii microscopice a electronului de oligonucleotide, colorate cu nanoparticule.

Figure 7. Transmission electron microscopic image of oligonucleotide stained with gold nanoparticles.

4. CONCLUZIE

Chimia pe care o indicam pentru etichetarea ADN cu nanoparticulele de aur deschid calea pentru mai multe aplicatii, cum ar fi interclasarea ADN sau studiile pentru rezolutie ridicata a acizilor nucleic in microscopul de electroni. Nanoparticulele pot fi atasate in mod selectiv la grupul de zahar al nucleotidelor depurinizate prin aminaza de reductive. Procesul de depurinizare este specific, datorita faptului ca este conditionat de conditiile de

4. CONCLUSION

The chemistry we report for labeling of DNA with gold nanoparticles opens the way to many applications such as DNA-sequencing or high-resolution studies of nucleic acids in the electron microscope. The nanoparticles can be attached selectively to the sugar moiety of depurinated nucleotides by reductive amination. The depurination process is specific, because it strongly depended on

reactive si modificare a nucleobazei. AND-ul etichetat este stabil pentru o perioada indelungata in solutii de amortizare. Aceasta proprietate ofera un alt potential pentru aplicarea acestora in diagnosticul experimental, acolo unde este necesar acidul nucleic, colorat.

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reaction conditions and nucleobase modification. The labeled DNA is stable for long time in buffer solution. This propertiy gives another potential for their application in the experimental diagnostic, where stained nucleic acid is required.

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