

Chapter 6

Hybridization Techniques in Nanotechnology- Present State and Future Trends

Diana Gonciar¹, Lucia Agoston², Teodora Mocan³

¹Gastroenterology Institute; “Iuliu Hatieganu” University of Medicine and Pharmacy, 19-21 Croitorilor Street, Cluj-Napoca, Romania, tel/fax: +40264-439696

²Department of Medical Sciences, Iuliu Hatieganu University of Medicine & Pharmacy, 2-4 Clinicilor, 400006, Cluj-Napoca, Romania

³Department of Physiology, “Iuliu Hatieganu” University of Medicine and Pharmacy, 5-8 Clinicilor Street, Cluj-Napoca, Romania

The process of hybridization involves mixing two nucleic acids strands. The two strands are coming from two different molecules of DNA or RNA. To separate the chains, the molecules are usually treated with alkaline substances or heated. To identify and isolate the target nucleic acid, one of the chains is labeled with a radioactive tracer or a fluorescent compound, known as probe. The probe will reassociate with the target nucleic acid sequence by the complementarity of the bases principle (adenine is paired with thymine or with uracil and guanine is paired with cytosine) and the DNA or RNA chains may be simply detected. Based on this process, several laboratory techniques as fluorescence in situ hybridization, polymerase chain reaction, northern, western and southern blot were developed.

Fluorescence in situ hybridization (FISH) is a cytogenetic molecular technique, used to detect a specific sequence of DNA or RNA, usually in the packaged form (chromosome), but not necessarily. The probe is marked with fluorescent dye and it is complementary with the target DNA sequence which will hybridize to. It can be used to localize a gene position, for karyotyping, to detect the chromosomal abnormalities, gene-mapping, to mark the entire chromosomes, or to label the interphase DNA. As a disadvantage, FISH does not offer information about the cell type (1), but it brings the possibility to use simultaneously more than one probe to examine different targets (2).

Roger M et al. used polylactic acid nanoparticles (PLA-NPs) and lipidnanocapsuls (LNCs) to highlight if marrow-isolated adult multilineage inducible cells (MIAMI) can be used as a drug vehicle system in brain tumor therapy. They injected athymic mice with human glioma cells and then, MIAMI cells, loaded or not with PLA-NPs or LNCs, were injected in the tumor. The rodents were sacrificed. MIAMI cells were detected using FISH assay. Y-chromosome probe is employed. Unloaded MIAMI cells were localized between the normal and the affected tissue. No signals were seen in the normal brain and similar migration of the loaded MIAMI cells was observed. Authors concluded that MIAMI cells are veritable carriers for NPs in brain tumor (3).

Quantum dots (QDs), nanocrystalline semiconductors, have an advantageous photostability, longer fluorescence emission period (4) and have a narrow emission peak when are excited with different wavelengths (5). When are labeled with specific oligonucleotides, the nanoparticles can be used as veritable FISH probes and have the ability to reduce the signal/noise ratio (6).

QDs FISH probes designed by Y Choi et al are employed to target specific *Drosophila* Rp49 mRNA. To test the specificity, negative controls were performed. When human telomere specific DNA sequence was used, no signal was observed. By adding RNase A, the intensity of the fluorescent signal decreased. The FISH assay performed for *Dif* gene, suggested that QDs probes are able to identify low levels of mRNA. Lipopolysaccharide (LPS) induces transcription of the *Dpt* gene. To suggest the relation between intensity of the signals and the level of transcription, *Drosophila* cells were treated with different amounts of LPS. An increase of the intensity of the signals was observed when FISH was performed for the specific mRNA, suggesting that it can be used as a quantitative method. The authors also tested the ability to use two different QDs probes to target specific mRNA to assess the potential in multiplex imaging. The

signals for the specific Act5C and Rp49 mRNA were highlighted (7).

QDs based molecular beacon (QDs- based MBs) developed by SM Wu et al. were used to target the β -lactamase gene, located in the plasmid pUC18 in *Escherichia coli*. β -lactamase gene is involved in the antibiotic resistance. FISH was performed using the designed nanoprobe, which are able to preserve their bioactivity even reassociated with complementary DNA sequence. The authors observed that the background noise was eliminated and clearer focusing was achieved. The nanosystem described might be veritable FISH probe for detecting antibiotic-resistant bacteria because it has the ability to penetrate the cell, to hybridize with the target DNA and to generate specific and clear signals (8).

Bentolila and Weiss designed QDs based FISH probes, containing DNA oligonucleotides against minor and major mouse satellites. First, one type of probe was used to target the minor satellite of the mouse mast cells. To evaluate the specificity of this method, negative control was also performed, by adding the QD probe against mouse major satellite on human HeLa cells and an irrelevant probe. No signal was detected. Then, two different labeled probes were simultaneously added to target the major satellite. Co-localization was observed. As a conclusion, the designed FISH probes can be used in multicolor FISH assay with the advantage that only one excitation wavelength is required (6).

Chromogenic in situ hybridization CISH assay is a laboratory technique similar with FISH but it also brings the immunohistochemistry advantages, to visualize the tissue morphology. DNA probes, labeled usually with digoxigenin, are used to hybridise with the target DNA sequence. The primary antibody binds to digoxigenin and then the secondary antibody combined with peroxidase is added. The substrate for peroxidase is diaminobenzidine. Hematoxylin staining is then performed and the slides are analyzed using a dark-field microscopy (9).

Polymerase chain reaction (PCR) is a wide-used technique, based also on the hybridization process. It allows amplification of small DNA sequences which will be further used. PCR has a variable number of repeated cycles and each cycle contains three steps. First, the reaction mixture, composed by the target DNA, the primers, free nucleotides and the DNA polymerase is heated, to let the complementary strands to separate. Second, because of the temperature decrease, the primers will hybridize with the target DNA chains. On the third step, DNA polymerase will add complementary nucleotides to the forming-chains. These steps are repeated and the DNA is exponentially amplified. PCR can be used as a qualitative method and also as a quantitative one (qPCR). To determine the amount of RNA, reverse transcriptase is required to transcript the information into a complementary DNA fragment. This method is known as reverse transcription PCR (RT-PCR). (http://www.roche.com/pcr_e.pdf) Quantitative real time PCR uses fluorescent dyes to keep under observation in real time the total amount of the probe. First used fluorescent dye was ethidium bromide (Higuchi et al (10)), but SYBR Green I is currently one of the most preferred fluorescent dye

Exosomes are nano-scale vesicles, secreted by all type of cells, in vivo and in vitro, in both normal and affected cells. The vesicles contain RNA and proteins and are found in body fluids. Exosomes were isolated from the serum of patients with metastatic prostate cancer, who have undergone prostatectomy and from patients without any history of prostate cancer, serving as control (M Li et al). miRNA was isolated from the nano-vesicles and then qRT-PCR was performed and the cycle threshold (Ct) values were determined. The Ct values corresponding to the twelve amplified types of miRNA were compared and it was revealed that no significant difference was observed between the control and the prostate cancer group related to miR16, 20a, 96, 107, 141, 145, 183, 221 and 409. For miR21, 375 and 574, the Ct values were lower for the patients

with prostatectomy than the others from the control group, but higher than the values from the prostate cancer group. Considering the inverse proportionality relationship between the Ct value and the amount of specific amplified miR, the authors suggested that miR21, 375 and 574, which are founded to be in higher levels in the serum samples from the prostate cancer patients, associated with exosomes nano-vesicles, may be veritable markers in prostate cancer (11).

R Say et al. synthesized Taq DNA polymerase nanoparticles and compared them to the traditional DNA polymerases. PCR assay was performed to emphasize the properties of the new enzyme. The reusability of the developed-enzyme was suggested by recovery the nano- polymerase using centrifugation. Taq DNA polymerase was reused and, after 5 cycles, the enzyme loss of activity was small. Compared to the traditional enzyme, the synthesized one has a higher stability against chemical and physical agents, due to the cross-linked intrinsic structure and also, a small quantity was required to perform the PCR assay. The nano-polymerase developed by the authors provides a new perspective of an economically and quicker PCR assay because of the important properties presented (12).

Silica-based nanoparticles, developed by Y Guo et al. are coated with streptavidin and then, are labeled with specific 5'-biotin- oligonucleotides. The nanocomplex is designed for hepatitis B virus (HBV) covalently closed circular DNA (cccDNA), which is a considered to be a valid marker for HBV infection. The authors assessed the performance of a quantitative assay for cccDNA, based on based on magnetic capture hybridization and quantitative PCR (MCH-qPCR).

The proposed-method was compared with real-time PCR and it was shown that combining the MCH, the specificity of the method increased. The increase of specificity resulted from the increase of amplicon in serum of HBV infected patients, when it was quantified without the MCH step. The amount of cccDNA

detected, was close to the total amount of HBV DNA, suggesting the non-specific amplification process. The cccDNA could not be quantified from all the serum samples because one of them has a low-concentration of DNA. Between the cccDNA log₁₀ concentrations and the log₁₀ expected concentrations a good linearity relationship was identified, suggesting that the introduction of the MCH step could increase the parameters of the detection assays for cccDNA (13).

KP Miller et al. designed silicon dioxide nanoparticles (Si-NP) to reduce bacterial communication. To achieve the aim of the study, *Vibrio fischeri*, Gram-negative bacterium, was used. It has the property to generate bioluminescence, via the lux operon. N-acyl-L-homoserine lactone (HSL) is a signaling molecule generated by Gram-negative bacteria, able to activate the bioluminescence process. The nanoparticles were coated with β -cyclodextrin (β -CD) because HSL is allowed to bind to β -CD. The bacteria were treated with the proposed biosystem. To assess the efficiency of SI-NP in silencing bacterial communication, qPCR was performed to detect the difference between transcription of the lux operon in the treated group and in the control group. Results show that the proposed nanosystem decreased transcription of the lux operon, which indicate that Si-NP is able to down regulate bacterial communication (14).

X Hu et al developed doped-fluorapatite (FA) with Yb³⁺ and Ho³⁺ and then, coated the nanocrystals with dextran. The described nanoparticles were tested for the ability of labeling and tracking the chondrogenic differentiation. To achieve the purpose, rat bone marrow stem cells (BMSCs) with the ability to secrete constant amount of green fluorescence proteins (GFP) were employed. BMSCsGFP were treated with different amounts of nanocrystal and chondrogenic differentiation was induced. Gene expression of Sox9, Col II was followed by real-time PCR analysis and it was shown that the transcription level

did not change. Based on these data and also on the evidence that no difference was observed when cells treated with nanocrystals were stained with alcian blue after 21 days of chondrogenic differentiation induction, the authors concluded that treated BMSCsGFP are able to differentiate in vitro into chondrocytes (15).

GR Beck et al. performed RT-PCR to demonstrate that silica nanoparticles-NP1 do not elicit an inflammatory response. The murine monocyte cell lines (RAW264.7) were treated with NP1 or with the polyethylene glycol (PEG) coated NP1. It was shown that the nanoparticles employed do not initiate the inflammasome based cleavage of the precursor of IL-1 β when compared with the non-treated cells group. A positive control test was performed by treating RAW264.7 with LPS, a molecule able to generate IL-1 β . RT-PCR assay showed that LPS induced IL-1 β transcription. The authors concluded that the nanoparticles had no consequences on the immune system activation (16).

MN Weitzmann et al. employed silica-shell nanoparticles, combined them with magnetic cobalt ferrite nanoparticles (MNP) and coated with PEG. The described nanosystem was used to assess the potential to induce differentiation of the osteoblasts and to promote bone mineralization in vivo. To validate if NP1-MNP-PEG specifically influence mineralization on osteoblasts, qRT-PCR for ALK, OSC and Runx2 mRNA was performed on human aortic smooth muscle cells (HASMCs) and human embryonic kidney cells (HEKs), treated with the described nanoparticles. It was shown that no mineralization response was founded on HASMC and HEK, compared to osteoblasts, which proved a good response as a result of the treatment. The proposed nanoparticles are efficient in the specific induction of mineralization and differentiation of the osteoblast cells (17).

Gold nanorod-siRNA nanoplex (GNR-siRNAD) employed by AC Bonoiu

et al. was used to investigate the interference with DARPP-32 (adenosine 3',5'-monophosphate-regulated phosphoprotein) gene silencing in dopaminergic neuronal cells (DAN). DARPP-32, key protein in DAN cells, is activated by opiate addiction. DAN cells were treated with GNR-siRNAD and q-PCR was performed to evaluate the levels of PP-1 and ERK, effectors of the DARPP-32 signaling pathway. It was observed that the two proteins significantly decreased in the presence of GNR-siRNAD, which may indicate the potential usage of the proposed nanosystem in addiction therapy (18).

Southern Blot is a laboratory method used to detect DNA molecules from a mixture. The sequences are separated using electrophoresis, according to their size and then, are transferred on a blotting membrane and are let to hybridize with specific DNA probes, which are labeled with fluorescent or radioactive compounds. (<http://www.nature.com/scitable/definition/southern-blot-289>).

Cationic gold nanoparticles were developed to form a gene carrier complex (T Niidome et al). They were loaded with plasmid DNA containing the luciferase gene and then HeLa cells were treated with the proposed gene delivery system. Southern blotting was performed to determine the amount of DNA transfected into cells. It was observed the correlation between the amount of DNA and the expression level of luciferase and also, a significant proportion of gene transfection was succeeded. The present nano-delivery system may be a veritable compound of a future gene therapy (19).

ES Hwang et al employed single walled carbon nanotubes (SWCNTs), which advantageous optical properties for bioimaging, labeling and tracking- the nanotubes do not blink and are able to eliminate photobleaching. SWCNTs express stable Raman scattering. Based on these data, the authors conjugated SWCNTs with specific single-strained DNA oligonucleotides, complementary with the DNA sequences of Escherichia coli, used as a control, and human

fibroblasts. Southern blotting assay was adjusted and Raman scattering was used instead of fluorescent staining. It was observed that the method is sensitive and specific. G peaks had lower amplitude when the amount of target DNA decreased. The borderline of detection the target DNA was evaluated to be 0.18 pg μl^{-1} which may suggest that Southern blot was improved by using SWCNT probes and it has achieved a comparable sensitivity with PCR. Future perspective of the technique includes adjusting the nanotubes length because DNA sequences may bind to them, instead of hybridizing with specific nucleic acid sequences (20).

Northern blot derives from Southern blot analysis and it also used to detect specific nuclei acid from a mixture of molecules. In this case, the nucleic acid is RNA. It requires electrophoresis separation, transferring on a blotting membrane and labeled specific probe to detect the RNA sequence. (<http://www.nature.com/scitable/definition/northern-blot-287>)

GR Beck et al. used Northern blot to determine the expression of characteristic osteoblast genes in pre-osteoblastic cells (MC3T3), fibroblastic cells (NIH3T3) and RAW264.7 cells, treated with NP1. The nanoparticles were also validated by PCR to determine if they are able to elicit an immune response. It was founded that NP1, after 7 days of treatment, increase the expression of sialoprotein, osteopontin, osteocalcin and osterix in a dose dependent manner in MC3T3 cells. In non-osteoblastic cells (NIH3T3 and RAW264.7), no significant effect related to the upregulation of the bone matrix proteins was observed, which may indicate that NP1 promote specific differentiation. The authors concluded that presented nanoparticles are able to enhance transcription of specific osteoblast genes in MC3T3 cells, promoting the differentiation process (16).

Y Peng et al. designed an electrochemical biosensor for direct detection of miRNAs. The action process of the nanocomplex is based on the deposition of polyaniline after the proposed system hybridize with the target miRNA. The

deposition is determined by RuO₂. Northern blot was performed to evaluate the capacity of the proposed biosensor to identify let-7 miRNAs. To achieve the aim, HeLa and lung cancer cells were employed. The results showed that specific miRNA was founded to be in a lower level when compared to normal cells. The method promotes a high sensitivity because the borderline of detection was founded to be 1 ng of RNA for less than 100 cells (21).

Mice experimental Ewing sarcoma was achieved by A Maksimenko et al. in order to emphasize the effect of antisense oligonucleotides (AON) loaded in nanospheres or nanocapsules against EWS-Fli-1 oncogene. After the intratumorally injection with AON free or loaded, the tumor were removed and northern blot was performed. It was revealed that both nanodelivery tools were efficient in release AON and the presented nanosystem is able to elicit specific down regulation of EWS-Fli-1 mRNA, indicating the role as feasible agents in Ewing sarcoma and also as effective delivery systems (22).

The development process of identifying RNA illustrates a trend towards inquiring into nanoparticles properties. Several assays for detecting specific miRNA were developed with the involvement of nanoparticles-based probes, optimizing the sensibility, specificity, reducing the background artifacts and providing time-saving detecting methods (23-25).

Western Blot combines the advantages of electrophoresis and the specificity of detection brought by the usage of antibodies to attain a precisely method to identify and quantify the amount of proteins. After the separation of proteins via electrophoresis, according to the molecular weight and to the isoelectric point, they are transferred on a membrane on which specific antibodies for the target proteins are added to be easily identified. The thickness of the bands seen are proportional with the amount of protein detected (26).

To relieve the usage of cationic albumin-conjugated pegylated nanoparticles (CBSA-NP) as an effective gene delivery system, western blot assay was performed by W Lu et al. The novel nanosystem was preferred to be used in gliomas because it can cross the brain-blood barrier as compared to the viral vectors. It was loaded with plasmid Porf-hTRAIL which encodes Apo2L/TRAIL to induce a selective apoptotic pathway. Mice were treated with C6 glioma cells and divided in three groups, one injected with CBSA-NP-hTRAIL, the other one with NP- hTRAIL, and the third one was used as control. The highest protein level was detected in the second day after treatment and it remains raised in the sixth day after the injection. The proposed system may be a feasible agent in glioma therapy (27).

Amine-modified single walled carbon nanotubes (a-SWNTs) were engineered by HJ Lee et al to scrutiny the implications in stroke. To achieve the purpose, rats were employed and divided in two groups, one pre-treated with a-SWNTs and the other with PBS. Following the administration, brain ischemia was induced by middle cerebral artery occlusion surgery (MCAO). One week after the procedure, tissue from the ischemic area was used for western blot. On a first step, blots were immunoprobed for p53 and Bax and it was revealed that the expression level was downregulated in the treated group. P-ERK, a kinase involved in cell survival and a regulatory factor for apoptosis process, was higher in the control group than in the treated group, result which may indicate that the infarct area was larger in PBS-group. P-Akt expression level was founded to be comparable for both groups. Moreover, rats received an injection with LY294002, an antagonist for Akt and it conducted to the death of PBS-treated rats after the MCAO procedure. The finding conducts to the conclusion that Akt is a key component in the limitation of brain damage. In addition, N-Cad levels were higher in a-SWNTs treated rats, indicating that the protective effect is realized by sustaining cellular adhesion. Moreover, HIF-1 α ,

VEGF (involved in angiogenesis) and DCX (neurogenesis marker) were downregulated in treated group, sustaining the conclusion that a-SWNT pre-treatment reduce the brain injury amplitude which leads to a diminishment of the angiogenesis and neurogenesis processes (28).

Y Huang et al. tested the efficiency of SeNPs coated with transferrin and loaded with doxorubicin on MCF-7 (breast adenocarcinoma human cells) as a potential anti-tumor agent. After the cellular uptake of the developed nanosystem, western blot was performed to establish the apoptotic pathway activated. Results show an upregulation of the histone phosphorylation which indicates that the DNA is damaged, so it may induce apoptosis via p53 pathway. The upregulation of the phosphorylated p53 is also registred and demonstrates the implication of p53 in the apoptotic process induced by the proposed nanosystem, which is also sustained by the MAPK signaling cascade. Western blot was also performed for the in vivo study and results suggested the correlation with the MCF-7 experiments. Authors concluded that the novel nanosystem may be successfully used in cancer therapy and it was shown that it is able to activate p53 mediated apoptosis (29).

Silica NP1 employed by GR Beck Jr et al were added on MC3T3 and western blot was performed to evaluate the capacity of suppression the osteoclastogenesis function. It was found that the nanoparticles are able to prevent activation of subunits p50 and p52 of NF-Kb, which indicates that silica NP1 suppress differentiation of osteoclasts. Western blot assay was performed for Runx2 level in MC3T3 cells treated with NP1 and it was shown that the nanosystem upregulated the expression of the transcription factor, which is involved in the differentiation process of the osteoblasts. Taking into consideration the results validated by PCR and northern blot, the authors concluded that NP1 promise an effective agent against bone loss (16).

As the drug resistance phenomenon is one major cause of failure in cancer therapy, novel strategies are developing to fight against it. Based on the fact that tumor cells often have an increased number of folate-receptors, T Liu et al coated SeNP with folate and added a payload of ruthenium polyridyl (RuPOP) with fluorescence and anticancer activity. Western blot was used to evaluate if the level of folate receptors (FAR) interfere with the cellular uptake because it was observed that R-HepG2 exhibited the strong fluorescence at the same moment, when compared to HepG2 and L02 cells treated with FA-SeNp. Results revealed that the FAR level was the highest in R-HepG2. The authors performed western blotting for the ABC family in R-HepG2, HepG2 and L02 cells and observed that the highest expression level is in R-HepG2. Furthermore, the assay was performed in R-HepG2 cells treated with different concentrations of FA-SeNPs and it was revealed the downregulation of ABC family related to concentration of nanoparticles used, result which lead to the conclusion that the proposed nanodrug may prevent drug resistance. To examine the anti-tumor effect, western blot assay for caspase 3, caspase 8, caspase 9 and PARP revealed that both intrinsic and extrinsic apoptotic pathways were activated. Moreover, the increase of p53, Ser 15, p-ATM, p-BRCA1, H2A.X, p38 and JNK reinforce the idea that FA-SeNp promotes apoptosis via p53 signaling cascade (30).

P Liu et al employed an efficient drug delivery for cancer therapy, based on amphiphilic graft copolymer poly (lactic-co-glycolic acid)-g-dextran (Dex-PLGA). Nanoparticles were loaded with paclitaxel (Dex-PLGA/PTX). P-gp is an efflux pump and it is often overexpressed in the membrane of cancer cells, phenomenon which leads to drug resistance. After the uptake of the presented nanodrugs into MCF-7(breast cancer cells) and MCF-7/Adr (multidrug-resistant breast cancer cells), western blotting was performed to evaluate the level of P-gp to demonstrate if overcoming drug resistance is dependent on downregulation P-gp. Without the uptake of Dex- PLGA/PTX, it was shown that MCF-7 cells do

not express P-gp. After the treatment with Dex- PLGA loaded or not with PTX, western blot assay indicates that P-gp level was not affected. Moreover, flow cytometry sustains the results and lead to the conclusion that the developed nanosystem do not overcome drug resistance by reducing the P-gp levels (31).

GNR-siRNAD employed by Bonoiu et al was also validated by western blot to scrutiny the effect in addiction. After the uptake of the present nanoparticles in DAN cells, DARPP-32 level was quantified and it was observed that the expression level decreased. The result lead to the conclusion that the proposed nanosystem enhance gene silencing. Moreover, PCR testing straighten the idea that GNR-siRNAD may be successfully used in addiction treatment (18).

In order to assess the effect of MWCNTs on DNA, mouse embryonic stem cells were employed by L Zhu et al and treated with present nanotubes. Blots were immunoprobed for Oct4 both in treated and untreated cells. The elevation observed in treated cells suggests that MWCNTs initiate cell differentiation. An increase in a dose-dependent manner was observed in treated cells regarding to the p53 expression levels, which indicates that the nanotubes elicit apoptosis. It was founded that MWCNTs also promotes p53 phosphorylation. Furthermore, the increase of both isoforms of 8-oxoguanine-DNA-glycosylase1 (OGG1) was observed, indicating that MWCNTs cause damage in nuclear and mitochondrial DNA by a guanine lesion. Expression levels of Rad51 and X-ray cross-complementation group 4(XRCC4) were also founded to be elevated in treated cells, revealing that nanotubes induce double stranded DNA subsequent breakdown. Moreover, SUMO (small ubiquitin-like-modifier) -modified XRCC4 expression level was increased. MWCNTs were founded to cause DNA damage, conclusion that enhances the importance of analysis the toxicity of nanomaterials (32).

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