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Meeting Report: 2016 RNA Nanotechnology Conference – Fusion Conferences Limited

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It is evident now that in the past few years the field of *RNA nanotechnology* (1-3) has expanded tremendously, bringing under its umbrella a large group of interdisciplinary sciences and researchers with different areas of expertise. The most recent RNA nanotechnology conference organized by the Fusion Conferences Limited was held August 1–4, 2016 in Wokefield Park, Berkshire, United Kingdom. This was the fourth meeting in the *RNA Nanotechnology and Therapeutics* conference series following the highly successful international meetings held in 2010 (Cleveland, OH, USA), 2013 (Lexington, KY, USA), and 2015 (Gordon Research Conferences, Ventura, CA, USA). The first meeting in Cleveland, nicely summarized by a review paper published in *ACS Nano* (4), drew the attention to the growing field of *RNA nanotechnology*. The second successful and well-attended conference in Lexington (5) paved the way to organizing the follow up annual meetings that involved a broader range of scientists and experts with different research backgrounds. 2015 Gordon Research Conference had a great recognition with the discussed topics as outlined in the conference webpage. The most recent meeting in Berkshire was also chaired by Dr. Peixuan Guo (The Ohio State University, USA), who pioneered the field of RNA nanotechnology, and co-chaired by Drs. Eric Westhof (Université de Strasbourg, France), Xing-Jie Liang (National Center for Nanoscience and Technology, China), and Bruce Shapiro (National Institutes of Health/National Cancer Institute, USA. To promote RNA nanotechnology interests across the globe, the United Kingdom, a scientific hub in Europe, was chosen to host this meeting. The field of RNA nanotechnology is a multidisciplinary field that has participation from scientists of varying backgrounds. Therefore there is often a gap in shared knowledge between the disciplines. The primary goal of the conference was to engage in proactive discussions within a small group of recognized inter-disciplinary experts (in chemistry, biochemistry, structural biology, microbiology, cancer biology, cell biology, biophysics, pharmaceutical sciences, material sciences, nanotechnology, and engineering) on how RNA can be utilized as a nanomaterial to make substantial leaps in the nanobiotechnology frontier. Furthermore, the conference provided not only an educational platform to disseminate knowledge, but also promoted new collaborations between scientists in different subcategories of RNA nanotechnology to grow and expand the field. This report aims to summarize several key scientific breakthroughs and emerging cross-disciplinary approaches in the field based on the presentations and discussions, which can be grouped into five sub-themes as discussed in this report.

Keynote talks

The first keynote address was given by world renowned RNA structural biologist Dr. Eric Westhof. RNA can fold into intricate structures mediated by canonical and non-canonical base-pairing, base stacking and tertiary interactions. To understand the folding of RNA motifs, it is necessary to dissect the rules that govern base pairing. There are 12 geometric base pair families for RNA with the canonical Watson-Crick base-pairing being only one of them. Dr. Westhof delineated the important roles of the other 11 non-canonical base pairs as well as several architectural modules in RNA secondary structures. These fundamental studies continue to lay the groundwork for constructing RNA nanoparticles with desired functions *de novo*, by modulating the twist between co-axially stacked helices, introducing stabilizing interactions mediated by

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ions, manipulating small molecule or protein binding sites, and exploiting some chemical and geometrical constraints for assembling RNA architectures. The second keynote address was by Dr. Kirill Afonin (University of North Carolina at Charlotte, USA), who has been developing dynamic RNA nanotechnology approaches. One of the major challenges in the field is to conditionally activate multiple functionalities inside targeted cells. Dr. Afonin presented recent advances in developing a catalog of RNA, DNA and RNA/DNA hybrid nanoparticles of different shapes to activate multiple functionalities such as RNA interference (RNAi), Förster Resonance Energy Transfer (FRET), functional aptamers, and transcription in response to external factors. The thermodynamic and kinetic properties of the nucleic acid nanoparticles can be easily fine-tuned by altering the primary sequence compositions. This concept served as the basis for the design of self-recognizing nucleic-acid-based shape switching nanoparticles.

Structure and folding of RNA nanoparticles

Similar to the recognized challenge with protein structure predictions, the prediction of RNA folding is not a trivial task. There is clearly a gap in our understanding of the link between RNA sequence and RNA 3D structures. Several groups are trying to dissipate this challenge. The most recent advances were presented by several well-recognized experts of RNA folding. Drs. Neocles Leontis and Craig Zirbel (Bowling Green State University, USA) have developed tools for RNA 3D structure prediction and RNA designs by looking at the sequences and geometry of RNA hairpins and internal loops (RNA 3D Motif Atlas). The Atlas demonstrates that different sequences can make the same 3D geometry, and same sequences can make different geometries in different contexts. The Atlas is currently being extended to cover multi-helix junctions. *In vitro* evolution has emerged as an efficient method for selecting novel RNA motifs with diverse functions such as cell binding, catalysis and fluorescence activation of small molecules. Dr. Michael Ryckelynck (Université de Strasbourg, France) introduced an innovative high throughput screening methodology based on droplet-based microfluidic technologies for isolating RNA motifs with optimized properties. The k-turn is a widespread motif in many RNA molecules, it generates a kink in RNA helices and mediates long-range tertiary interactions in folded RNAs. Dr. David Lilley (University of Dundee, UK) elucidated a series of rules that govern k-turn folding and its utility to form dumbell, triangle and square shaped RNA nanoparticles with and without proteins bound to them. Dr. Thomas Hermann (University of California, San Diego, USA) discussed crystal structure guided designs of 2D (triangle, square) and 3D (cage-like) circularly closed RNA nano-objects using minimal number of RNA sequences that by themselves do not adopt a stable structure. Dr. Peixuan Guo demonstrated the utility of the bacteriophage phi29 packaging RNA three-way junction motif (pRNA-3WJ) with controllable angles and arm lengths to construct 2D and 3D architectures with various shapes and sizes. The arrays of pRNA-based nanoparticles provide a rich source of materials for biodistribution profiling of RNA nanoparticles in cancer mouse models. Steffen Sparvath from Dr. Ebbe Anderson's group (Aarhus University, Denmark) presented their work on constructing 3D RNA origami constructs for potential applications in metabolic engineering and intracellular sensing. A 6-helix bundle structure was designed using intra-connecting kissing loops that enabled the bundles to be folded from a single strand of RNA as they are transcribed, while dovetail seams determined the overall arrangement of the helices. DNA nanotechnology has been used to construct complex geometrical DNA architectures and some of the assembly principles based on topological routing of paranemic DNA crossovers can be applied for RNA nanotechnology, as delineated by Dr. Hao Yan (Arizona State University, USA). Complex DNA origami constructs can be made from a long single-stranded DNA or RNA without using traditionally short staple strands. These nanostructures provide opportunities for replication and amplification by biological machineries. In a related talk, Dr. Peng Yin (Harvard University, USA) discussed advances in developing digitally programmable DNA and RNA nanostructures interfaced with other functional modules such as DNA/RNA probes with optimized binding specificity and RNA-based translation regulators with wide dynamic range and othogonality.

RNA computation and modeling

Computational approaches are of paramount importance for predicting the 3D structures of RNA from primary sequences as well as for designing RNA sequences to fold in specific structures. Carefully developed *in silico* approaches that are in agreement with experimental data can assist in design and characterization of multifunctional RNA nanoparticles and their further biomedical applications. Dr. Shapiro's group has made substantial advances in this context over the years. Their most recent development is an algorithm, HyperFold, that is capable of predicting multi-strand RNA/RNA, RNA/DNA and DNA/DNA secondary structures. The utility of

HyperFold was demonstrated by designing RNA toehold interactions for conditional activation of hybrid nanorings and a two-stranded RNA switches in cells. This concept is useful as it allows different functional modules, such as RNAi inducers, to be activated in diseased cells in response to the internal stimuli. In order to understand DNA or RNA structure, dynamics, and function, one critical structural and energetic component is the ion atmosphere that surrounds the nucleic acids. Magdalena Gebala (Stanford University, USA) has developed an experimental approach using buffer-exchange atomic emission spectroscopy to quantify both the number of cations associated with and the number of anions excluded from nucleic acids. The results are in strong agreement with Poisson Boltzmann theory and other computations models. Dr. Shi-Jie Chen's group (University of Missouri-Columbia, USA) has developed new computational tools for quantitative modeling of ion effects and co-transcriptional kinetics frequently observed in RNA folding. This tool is useful for designing RNA nanoparticles with predictable folding motifs *in vivo* after transcription. Since RNA is known to regulate diverse biological processes, it has become imperative to develop specific RNA-binding small molecules for therapeutic intervention. Stuart Le Grice's group (NIH/NCI, USA) has developed a high throughput small molecule microarray screening approach to rapidly identify biologically active RNA-binding small molecules.

RNA nanoparticles in cellular reprogramming and therapeutics

It is technically challenging to access the intracellular information for distinguishing between target cell types. Rationally designed programmable RNA nanostructures can offer the unique advantages in addressing this challenge. Dr. Hirohide Saito (Kyoto University, Japan) designed synthetic mRNAs encoding a protein of interest tagged with target sequences of miRNAs. These synthetic miRNA switches are capable of identifying specific cell populations with high efficiency and accuracy, including cardiomyocytes, hepatocytes, insulin-producing cells, and human induced pluripotent stem cells. Detection of low abundance miRNA is important for cancer therapy since certain miRNA expression are known to regulate chemo-resistance. Dr. Jeong-Woo Choi (Sogang University, S. Korea) has developed a surface enhanced Raman spectroscopy-based intra-cellular detection method to detect *in vivo* miRNA in single cell level with high sensitivity. Dr. Hua Zhu (The Ohio State University, USA) discovered several miRNAs, such as miR-181a, that are dysregulated in muscular dystrophy. Dr. Subba Palli (University of Kentucky, USA) discussed advances in applying RNAi modules in agriculture and pest management and how nanotechnology can overcome the challenges associated with RNA instability and inefficient intracellular transport. Dr. Shuo Gu (NCI, USA) discussed important aspects of designing RNAi modules such as short hairpin RNAs (shRNAs) with optimized efficacy while minimizing unwanted off-target effects. Recent studies indicate that in addition to certain structural features of shRNA for precise processing by Drosha and Dicer, lower GC content in both 3'-regions of guide strand RNA and seed regions elicited minimal off-target effects while maintaining intended on-target effects. Cancer immunotherapy is rapidly emerging and approaches to regulate the innate immunity, natural killer immune cells and tumor development are necessary. MiRNAs can play extrinsic and intrinsic roles in this context. These aspects were delineated by Dr. Jianhua Yu (The Ohio State University, USA). Drs. Jayden Smith from Stefano Pluchino's lab (University of Cambridge, UK) discussed advances in using RNA nanoparticles harboring small interfering RNA (siRNA) for brain repair in syndromes where inflammation leads to chronic neural degeneration such as multiple sclerosis, cerebral stroke and spinal cord injury. Targeted delivery of RNA therapeutics is of paramount importance and cell-internalizing RNA aptamers are being rapidly developed through the *in vitro* screening method SELEX. Dr. Jiehua Zhou from Dr. John Rossi's group (City of Hope, USA) has developed a series of RNA aptamers (gp120, CCR5 or CD4) conjugated to siRNA (such as, LTR-362) for targeted HIV-1 therapy. A major challenge lies in the construction of containers with defined shape, size, and stoichiometry to load and deliver high doses of therapeutics such as native RNAs, DNAs, peptides, or drugs for controlled release in the human body. Danny Jasinski and Dr. Farzin Haque from Dr. Peixuan Guo's lab reported the use of a pRNA-3WJ motif as a versatile scaffold for constructing 3D tetrahedral and prism-shaped RNA containers as well as globular 3D shaped RNA dendrimers for encapsulating native therapeutic cargoes as well as for increasing the therapeutic payloads per nanoparticle. In a related work, Dr. John Bum Lee (University of Seoul, S. Korea) reported the construction of pre-programmed polymeric RNA sequences that spontaneously assemble into RNA microspheres, RNA membranes, and hydrogels for delivering high doses of RNAi or small drugs to cells. Dr. Yizhou Dong (The Ohio State University, USA) has developed a series of lipid-like nanoparticles for delivering messenger RNAs (mRNAs) into cells for transient expression of desired functional proteins for therapeutic intervention. The pRNA-3WJ based

nanoparticles are thermodynamically and chemically stable, and can be easily functionalized with targeting and therapeutic RNA modules simply by sequence integration. This is exemplified by Dr. Dan Shu's talk (The Ohio State University, USA) reporting the use pRNA-3WJ harboring EGFR targeting RNA aptamers and 8-nt anti-miRNA locked nucleic acid (LNA) fragments for suppressing triple negative breast tumors upon systemic injection in animal models. Similarly, Dr. Xiaoting Zhang (University of Cincinnati, USA) developed pRNA-3WJ harboring Her2 aptamer and siRNA (MED1) for targeted suppression of tamoxifen resistant Her2(+) breast cancer cells *in vivo*.

Physical and chemical approaches in RNA nanotechnology

Several RNA molecules function by rearranging their secondary structures upon binding of external factors such as proteins, ions or small molecules. These secondary structural switches often involve rearrangement of several base pairs in and around non-canonical base-pairs at second or slower timescales. Dr. Katja Petzold (Karolinska Institute, Sweden) presented advances in NMR methods for characterizing low abundance transient RNA structures observed in G–U wobble base-pair and HIV-1 dimerization initiation sites. Dr. Richard Lease (The Ohio State University, USA) has developed a high throughput genetic screen *via* rationally designed stem-loops for quantifying RNA regulatory activity. These genetic elements are under orthogonal, small-molecule induced transcription control. The system can potentially be applied for design and validation of ordered self-assembly of RNA nanostructures *in vivo*. Labeling of nucleic acids during *in vitro* transcription with high yield and precision remains a challenge. Dr. Carlo Montemagno (University of Alberta, Canada) has developed template-less enzymatic synthesis of oligonucleotides with high single nucleotide precision. Dr. Yitzhak Tor (University of California – San Diego, USA) reported advances in developing isomorphic fluorescent nucleoside analogues as versatile probes for real-time investigation of nucleic acids structure, dynamics, recognition and damage as well as metabolic processes. Dr. Hui Zhang (The Ohio State University, USA) reported the development of single molecule approaches for studying RNA nanoparticles. Single molecule photobleaching combined with binomial distribution analysis was used to determine the hexameric stoichiometry of the pRNA on active phi29 motors. A single molecule FRET studies were carried out to investigate the conformational change of the 3WJ motif at different Mg^{2+} concentrations. These single molecule assays can provide valuable insights to improve the designs, stability, and functionalities of RNA nanoparticles for various applications. Dr. Subha Das (Carnegie Mellon University, USA) described the use of conjugation strategies to specifically modify, label, and derivatize DNA/RNA-polymer hybrids as nanotags for imaging applications and as nanodelivery agents. Dr. Susan Schroeder's group (University of Oklahoma, USA) reported the use of NMR, fluorescence spectroscopy, crystallography, and optical melting to probe the structures, dynamics, and stabilities of 3WJ motifs derived from phi29, GA1, SF5 and M2 bacteriophages.

Nanotechnologies relevant to RNA research

More and more disciplines and technologies address their potential deficiencies and find their specific applications and uses within the field of RNA nanotechnology. Dr. Xiyun Yan (Chinese Academy of Sciences, Beijing, China) discussed advances in developing next generation artificial enzymes - termed nanozymes that are ferromagnetic nanoparticles with intrinsic peroxidase-like activity. Several variants of nanozymes are being developed for cancer diagnosis, tumor therapy and nanobiotechnological applications. Dr. Francesca Storici (Georgia Institute of Technology, USA) shared her recent exciting discovery that RNA is recombinogenic and can serve as precise template for chromosomal double-strand break repair *via* homologous recombination in yeast. These basic biological findings provide new avenues for designing therapeutic RNAs for genome engineering. Dr. Rob DeLong (Kansas State University, USA) discussed the development of inorganic/RNA complexes using zinc oxide/poly I:C and zinc oxide/Torula yeast RNA as anti-cancer models for developing therapeutic strategies against melanoma and other solid tumors. Dr. Xing-Jie Liang discussed advancements in non-viral nanoparticle designs and methods for tuning the physiochemical properties (size, shape and surface charge) for delivery of RNAi therapeutics to diseased cells with high efficacy while reducing toxicity and side effects.

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Nanotechnology, *RNA Biology*, *Kenkyu*, *Transcription*, *Select Science*, and *Haptic*.

Further reading: Readers are encouraged to read the following comprehensive sources to learn more.

1. Guo P. The emerging field of RNA nanotechnology. *Nature Nanotechnology*. 2010; 5(12):833-42.
2. Methods in Molecular Biology Series - RNA Nanotechnology Methods and Protocols (Humana press) (Ed: Peixuan Guo and Farzin Haque) 2015.
   - This book represents a comprehensive compilation of laboratory protocols relating to construction, purification and characterization of RNA nanoparticles for applications in nanotechnology.
3. RNA Nanotechnology and Therapeutics (CRC press) (Ed: Peixuan Guo and Farzin Haque) 2013.
   - This book details advances and challenges in RNA nanotechnology field for nanomedicine applications.
4. Shukla GC, Haque F, Tor Y, Wilhelmsson LM, Toulmell JJ, Isambert H, Guo P, Rossi J, Tenenbaum SA, Shapiro SA. A boost for the emerging field of RNA nanotechnology. *ACS Nano*. 2011; 5(5): 3405-3418.
5. Leontis N, Sweeney B, Haque F, Guo P. Conference Scene: Advances in RNA nanotechnology promise to transform medicine. *Nanomedicine*. 2013; 8(7):1051-1054.