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PROTEIN BIOCHEMISTRY, SYNTHESIS,
STRUCTURE AND CELLULAR FUNCTIONS

RECOLLECTIONS OF 45 YEARS IN RESEARCH: FROM PROTEIN CHEMISTRY TO POLYMERIC DRUGS TO THE EPR EFFECT IN CANCER THERAPY

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CONTENTS

PREFACE

In this book, I review recollections of my science career that spans almost half a century. During this period, all areas of the biological sciences—including biochemistry, immunology, pharmacology, genetics, cell biology, and oncology—have progressed beyond what one could have imagined 50 years ago, when many of these research areas were almost nonexistent or at an early stage of development.

Biological systems are, in fact, highly complex. As a consequence of multiple interactions within these systems, predictions in such systems are possible only for localized areas or small areas within the ordered complexity. One could, however, follow each evolving step in these systems and be able to deduce a reasonable conclusion or propose a plausible theory. For example, predictions concerning simple, localized in vitro phenomena that is occurring in model systems, are possible in many cases, such as the SCID mouse model, in which a host response such as an immune reaction is minimal if not totally absent. However, predictions of a comprehensive, complex system, such as carcinogenesis in humans, are almost impossible, particularly, which has a nature of unorganized complexity. This idea is similar to those presented by Friedrich August von Hayek in his lecture for the Sveriges Riksbank Prize in Economic Sciences in Memory of Alfred Nobel in 1974[1]. However, I believe that serendipitous findings (predictions) can have more value than findings obtained via rational routes. For students

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[1] From Friedrich August von Hayek—Prize Lecture. Nobelprize.org. 14 Sep 2011. Available at: http://www.nobelprize.org/nobel_prizes/economics/laureates/ 1974/ hayek-lecture.html.

of medicine, therefore, naive ideas are as important as or perhaps better than ideas proposed by an expert with a narrow specialty.

The integration of interdisciplinary knowledge or of the interactions of different disciplines becomes more important as medical science expands in various directions. This book demonstrates my understanding of this principle, as it covers my work in diverse research fields, from proteases to free radicals, protein conjugates to vascular permeability, and drug delivery to cancer treatment. Despite their superficial dissimilarity, all these topics are in fact ultimately interconnected.

As I look back over my career, which indeed continues today, I owe a great debt of gratitude to two of my mentors, Robert E. Feeney and Nakao Ishida, both of whom worked in different fields and with whom I spent the very early part of my career, when I was a graduate student. I am, of course, indebted to many more scientists, colleagues, and friends whose advice and suggestions were so valuable for me, my discoveries, and my hypotheses. In addition, although I cannot name here all the students who participated in and supported our research projects, their contributions were key to my achievements.

I must also give full credit to the support and sacrifices of my family, especially my wife Noriko Maeda, ever since I was a Ph.D. student at Tohoku University. Without their encouragement, I might have been much less productive, and much less satisfied, as well. I continually tell students that efforts expended during research are like compound interest: the more you work, the more you gain—not just a linear return but in fact, far greater long-term gains. The generous support of my family made such satisfactory returns possible.

I also had the good fortune to have excellent secretarial support throughout my career, as well as outstanding editorial assistance of Judith B. Gandy, who improved my manuscripts to the highest possible level. Without their assistance, handling the many issues that I faced within and outside the university, as well as my research activities, would not have been possible.

H. M

September, 2011, Kumamoto, Japan

INTRODUCTION

This article describes the lifelong research experience of a scientist, from the beginning, studies of protein chemistry to development of an antitumor protein drug (neocarzinostatin, NCS) to the invention of the first polymer conjugated drug—poly (styrene-co-maleic acid) [SMA] conjugated to NCS, called SMANCS. The author, having acquired knowledge of proteases and inhibitors, pioneered investigations of microbial proteases in the pathogenesis of bacterial infection. The author's group discovered an enormous burst in the generation of superoxide anion radical ($O_2^{\cdot -}$), using a polymer-conjugated enzyme (superoxide dismutase, SOD), during influenza virus infection which triggers the generation of xanthine oxidase. In this setting $O_2^{\cdot -}$ was found to be the major cause of the pathogenesis of this viral infection, which progressed even after the virus was eradiated. These events can be interpreted as advancing the boundary of Robert Koch's postulates, that is, *viral disease occurring in the absence of virus*. Also, the importance of the discovery of endogenous free radicals, now referred to as reactive oxygen species (ROS) and reactive nitrogen species (RNS), during the microbial infections. Role of ROS and RNS in human disease and health became clear, in that they were crucial factors for development of formation of mutant microorganisms (e.g. drug-resistant mutant) in chronic and acute infections as well as carcinogenesis.

In additional studies of the pathogenesis of infections of bacteria and fungi, activation of the kallikrein-kinin cascade and thus bradykinin (also called kinin) generation at the site of infection was found to result in pain and enhancement of vascular permeability (edema). Because no potent inhibitors of bacterial proteases exist in the human body, such proteolytic activity is detrimental to the host. The intense enhancement of vascular permeability of bacterial infection or inflammation was later found to be

analogous to the situation in cancer tissues. That finding led to the discovery of the EPR (enhanced permeability and retention) effect of macromolecules (>40 kDa) in solid tumors. This EPR effect can be utilized in the field of cancer chemo therapy that is today known as nanomedicine, for targeting of macromolecular anticancer drugs to tumors. When this type of drug is well developed one could avoid adverse effect and augment the therapeutic efficacy.

The EPR effect is now becoming a universal guiding principle for tumor selective targeting in nanomedicine for design of drugs such as polymer conjugates, liposomes, micelles, antibodies, and DNA/RNA-carrier complexes etc. The polymer conjugate drug SMANCS made remarkable pinpoint targeting possible with unprecedented selectivity: i.e., a tumor/blood ratio of drug concentrations more than 2000 was achieved. Further enhancement, 2- to 3-fold, of the EPR effect, and thereby more tumor selective drug delivery became possible via either angiotensin II-induced elevation of blood pressure or application of nitroglycerin ointment (which produces nitric oxide in tumors). The proof of concept of this therapeutic procedure was demonstrated for advanced cancers of the liver, kidney, bile duct, pancreas, lung in addition to the metastatic cancer; all these cancers are difficult-to-cure type cancer.

As described in the text, such interactions of multiple disciplines and of basic science and clinical problems have yielded many discoveries that will be invaluable to young scientists and future directions and development in the medical sciences.

Chapter 1

BON VOYAGE: PASSAGE TO THE MACROMOLECULAR WORLD AND PROTEIN CHEMISTRY

After obtaining my BS degree from Tohoku University, in Sendai, Japan, in March, 1962, I applied for an extremely competitive Fulbright Graduate Study Fellowship to study in the United States. With great luck, I was accepted into the Fulbright program for study at the University of California, Davis, for 2 years. There, I met Professor Robert E. Feeney, in August, 1962, for the first time; he was to become my mentor throughout my academic career. Professor Feeney, who passed away in September, 2007, was well known for his research on egg white proteins and, more specifically, on ovomucoids and other protease inhibitors. He may be better known, however, for his research on antifreeze glycoprotein found in blood plasma of Arctic and Antarctic fishes. He is also famous in the field of chemical modification of proteins. These research areas in fact became the basis of my own future research directions and developments.

At the University of California, Davis, my MS thesis concerned the molecular microheterogeneity of primarily the egg white protease inhibitors called ovomucoids with genetically identical backgrounds. The remarkable progress in protein chemistry and biochemistry during those days is comparable to that of genomic science at the end of the 20th century. My curiosity about the life sciences led me to choose medical science as my future interest.

Chapter 2

BACK TO SENDAI AND THE WORLD OF ANTIBIOTICS

After finishing my MS degree at Professor Feeney's laboratory, I returned to Sendai, Japan, which is about 350 km north of Tokyo, where I continued my studies at Tohoku University Medical School in Professor Nakao Ishida's Department of Bacteriology. When I had been an undergraduate, in 1960, I had the microbiology class that was taught by Dr. Ishida, who was then an associate professor at Tohoku University Medical School. He later became known throughout the world as the virologist who discovered Sendai virus, who passed away in December 2009 during preparation of this manuscript.

In Sendai, my first research project under Professor Ishida's supervision was to isolate interferon from the allantoic fluid of virus-infected embryonated chicken eggs. At that time, interferon was vaguely known as a protein, but not much more was known. World-class research projects, particularly studies of virus, were conducted in that department; among them, screening program for bioactive compounds with antibacterial, antiviral, or antitumor activity was also actively pursued.

By means of this screening program, Ishida's group discovered a unique antitumor protein, with unprecedented activity, which was named neocarzinostatin (NCS). Because of my former experience in protein chemistry, Professor Ishida assigned me to the investigation of the biochemical and chemical nature of NCS [1]. The biological activity of NCS were studied primarily by Katsuo Kumagai, MD, who had originally been a clinician but then a junior faculty member and later became Professor of Microbiology at Tohoku University School of Dentistry. The Southern Research Institute of the National Cancer Institute in the United States also

evaluated the antitumor activity of NCS in the murine Walker 256 tumor model and showed NCS to be one of the most active compounds evaluated among 50 or so new candidate drugs at that time.

To clarify the chemical structure of NCS, we initiated amino acid sequencing of NCS. Use of Sanger's reagent, dinitrofluorobenzene, that is coupled to *N*-terminal amino acid (to obtain dinitrophenylated derivatives), and Edman's degradation using phenylisothiocyanate was routine procedures. The automatic amino acid analyzer was also becoming a common tool in protein chemistry. In addition, dansyl derivatization with resultant higher fluorescence sensitivity, together with thin layer chromatography, became an accepted method for detection of N-terminal amino acid residues. Despite of inadequate resources, we began the research on amino acid sequencing of NCS. Meantime we developed a highly sensitive amino acid sequencing methods using fluorescein isothiocyanate (FITC), similar to the Edman reagent but much higher sensitivity, for which Hiroshi Kawauchi, a graduate student (a few year junior to me, who later became Professor of Kitazato University and one of the world most expert of the pituitary hormone of fish) contributed a lots, first by synthesizing pure FITC; commercial FITC at that time had a purity of no better than 60%, far from good chemistry. Although we published a few papers, the method did not become popular because no effort for commercialization was made, was one reason [2-4].

Any innovative project or large research undertakings required substantial funding. To obtain such research money, we applied for a research grant to the National Institutes of Health (NIH) of the United States. Several months after submission of the application to the NIH, we received a letter that our application was successful. However, our excitement was temporary. A few months after that letter, we received another letter from the Office of General Accounting stating that the grant could not be funded, perhaps because of the Vietnam War. Student sentiments against the Vietnam War were so strong that they would target the United States-supported research project, even though academic, with violence.

Chapter 3

ON TO THE UNITED STATES: WORK AT CHILDREN'S CANCER RESEARCH FOUNDATION AND HARVARD MEDICAL SCHOOL

Within a few months, Professor Ishida received a letter from Professor Sidney Farber, MD (1903–1973), who was the founder and Director of Children's Cancer Research Foundation (CCRF) of Children's Hospital Boston, at Harvard University Medical School; (CCRF is now Dana-Farber Cancer Institute). The letter stated that he and Dr. Meienhofer's group were interested in NCS and wanted to collaborate in research on NCS. The letter was accompanied by a letter from the late Johannes Meienhofer, PhD, at the same institute, who would be in charge of the project. Such an offer from Harvard was quite appealing, and Dr. Ishida decided that I should go to Boston next year, after my PhD degree was conferred.

Dr. Farber was a pathologist and pediatric oncologist who was well known throughout the world, as Professor at Harvard Medical School, and President of the American Cancer Society (1968–1969). He believed strongly that cancer chemotherapy would be effective, and he developed the concept of total care of pediatric cancer patients, which meant primarily free care and a wide range of support services at CCRF. He pioneered treatments of Wilms' tumor (a kidney cancer) of children with actinomycin D and of lymphoma and leukemia with methotrexate. Dr. Meienhofer was a synthetic peptide chemist working at CCRF, who had been trained in Aachen, Germany, and was involved in the organic synthesis of insulin under Dr. Helmut Zahn.

After I arrived in Boston in May, 1968, I started work on the amino acid sequencing of NCS by using the dansyl-Edman method. To gain more experience in amino acid sequencing, I was sent to the Hormone Research Laboratory of the University of California, San Francisco (UCSF), where Professor Chao Hao Li (1913–1987), who was a friend of Dr. Farber and a scientific consultant to CCRF, was the director. I spent about 2 weeks at the UCSF Hormone Research Laboratory near Golden Gate Park in San Francisco.

What I found was an unusual characteristic of NCS: it resisted chemical reduction of its two disulfide bonds, even under the excess amount of reducing agents, 5 M guanidine-HCl or 8 M urea. To reduce and alkylate NCS, I developed a new method of disulfide bond reduction in liquid ammonia with dithiothreitol, which worked perfectly; this method was published in 1971 in the *Journal of the American Chemical Society* [5]. The remainder of my work involved peptide fragmentation with trypsin, chymotrypsin, and thermolysin, and manual Edman degradation. At that time, high-pressure liquid chromatography for separating peptide fragments was not available, amino acid analysis was not completely automated, and 6 N HCl hydrolysis at 110 °C in vacuo was, of course, required. Tedious labor-intensive efforts were thus prerequisites for amino acid sequencing. About 1 year later, when the project was under way and productive, a postdoctoral fellow, Charles Glaser, joined in our group. When the major portion of the work was almost completed, Dr. Glaser went to UCSF for a second postdoctoral fellowship, and Kenji Kuromizu, another postdoctoral fellow from Kyushu University, Japan, joined our laboratory to help with this project. The total amino acid sequence was completed before long after his arrival [6-8]. The positions of the two disulfide bridges in NCS were determined later, after we returned to Japan [9], and a minor revision of the amino acid sequence [10] was also determined primarily by Dr. Kuromizu.

In the early summer of 1971, I received a letter from Professor Yorio Hinuma, MD, asking me to accept the associate professorship position at his Department of Microbiology at Kumamoto University Medical School. Dr. Hinuma became world known by his discovery of the human T-cell leukemia virus in later year, which is the causative agent of adult T-cell leukemia, for which he received the Hammer Award, among others. He also received the Order of Culture from the Emperor of Japan in 2009. His offer was a challenge for me, inasmuch as the amino acid sequence of NCS was almost completed, so I accepted the offer. I returned to Sendai in the middle of September, to finish work in my previous position, and then in October I moved to Kumamoto to begin work at my new position.

Chapter 4

KUMAMOTO UNIVERSITY IN KYUSHU, JAPAN

I had no acquaintances or relatives in Kumamoto, which is located in the central region of Kyushu Island. Kumamoto University Medical School is an old school with a long history that can be traced back to the 18th century. I continued my studies of NCS, such as chemical modification and structure-activity relationships, biochemical and molecular mechanisms of action, tissue distribution, and pharmacokinetics, pharmacodynamics, and degradation in vivo. One very interesting finding was the highly lymphotropic nature of NCS after subcutaneous injection. When ^{14}C-labeled succinylated NCS was injected subcutaneously, it accumulated primarily in regional lymph nodes [11]. Lymphatic tissue is known to be the preferred site of cancer metastasis, which is why lymphatic metastasis occurs so frequently. This lymphotropic drug accumulation motivated me to devise a strategy for treatment of lymphatic metastasis by utilizing this property: that is, delivering the drug directed to the lymphatic system. In fact, in lymphology, macromolecules and lipids were well known to be preferentially recovered by the lymphatic system after injection into the interstitial space. In other words, lipophilic and macromolecular derivatization of NCS would likely make such lymphotropic drug delivery possible, which would be ideal for an anti-lymphatic metastasis strategy. We also showed that, at a subcellular level, intracellular uptake was needed for DNA degradation by NCS. This finding meant that NCS activity occurred inside cells after entry by endocytosis, rather than signal modulation leading to NCS activity at the surface of the cell membrane [12-14]. Succinylation of NCS suggested that two of the free amino groups in NCS (one at the N-

terminal alanine 1 and the other at lysine 20) could be utilized for modification without loss of activity [15,16].

During this work, I found an advertisement in *Chemical and Engineering News* (a weekly journal of American Chemical Society) stating that poly(styrene-co-maleic anhydride) (SMA) could be used as car wax and floor polishing materials, which were water and solvent-soluble, hydrophobic polymers containing maleic anhydride. As was a case of succinic anhydride it would react with amino groups of NCS. Thus, I planned to modify two amino groups of NCS with SMA polymer. I expected that attachment of SMA to NCS would confer a potential (perhaps ideal) lymphotropic property to NCS. For this purpose, I asked ARCO <formerly Atlantic Richfield Chemical Co.> in Philadelphia for a small sample of SMA, and they generously sent me several 1-lb bottles of SMA with different specifications.

Without much difficulty, I reacted SMA (with a relative molecular mass M_r about 6 kDa) and NCS in bicarbonate buffer (pH 8.5), and purified the reaction product, then verified that two SMA chains were conjugated to NCS, which I named SMANCS (Figure 1). The first paper on the synthesis of SMANCS appeared in the *International Journal of Peptide and Protein Research* in 1979 [17]. Descriptions of the biological and pharmacological properties of SMANCS, including its highly lymphotropic nature, were subsequently published [18-20].

Because industrial-grade SMA was unfit for pharmaceutical development, I collaborated with the Kuraray Company in Kurashiki, Japan, which had experts in polymer chemistry and was highly interested in developing pharmaceuticals. After we compared various alkyl side chains of half-alkyl esters of maleic acid residues, we chose to work with the *n*-butyl half-ester of SMA for a number of reasons. The details of this synthesis and the chemistry of SMANCS were published in the *Journal of Medicinal Chemistry* in 1985 [21]. With the fully characterized polymer-conjugated antitumor protein SMANCS in hand, my graduate student Jiro Takeshita (who later became an anesthesiologist) and I were on board for a big voyage on the ocean of macromolecular pharmacology, or polymer therapeutics, as Professor Ruth Duncan later preferred to call it.

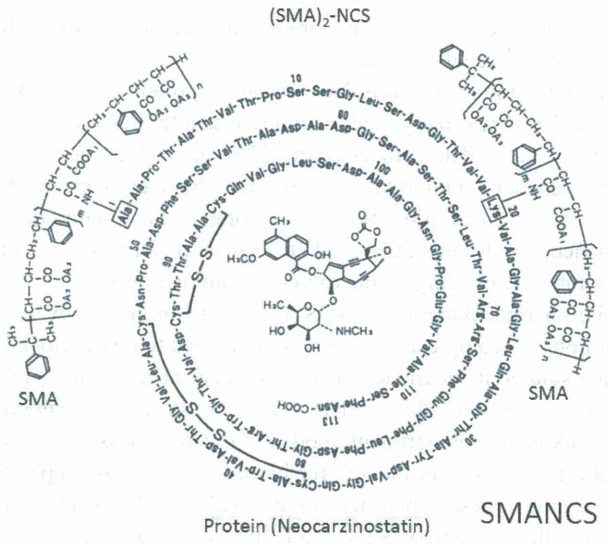

Figure 1. Chemical structure of SMANCS. SMANCS consists of three components: two chains of styrene-co-maleic acid polymer, a protein portion, and the chromophore of NCS.

In the late 1960s, the clinical development of NCS had started a phase I/II study in Japan, although anecdotal accounts of good clinical responses had previously confirmed its efficacy [22,23]. In this regard, my old close friend since college freshman in Tohoku University Medical School, Dr. Ryunosuke Kanamaru, who later became a professor of clinical oncology, was the first doctor to apply NCS in human that is now considered phase 0/I. Professor Nakao Ishida, who was the director of the project, collaborated with Kayaku Antibiotic Laboratories of Tokyo on the manufacturing of NCS and later with Yamanouchi Pharmaceutical Company (now Astellas Pharma Inc.) for marketing. After my return to Japan in 1971, I became a member of the NCS project and was often asked to give lectures on the chemistry, biochemistry, mechanism of action, pharmacology, and pharmacokinetics of NCS. The mode of action of NCS at the molecular level was first studied by my senior colleague, Yasushi Ono, MD, PhD, who demonstrated inhibition of DNA synthesis by NCS, and, at a higher dose, degradation of DNA as its primary mode of action. Dr. Kumagai explored the antitumor effect of NCS in vivo by using various murine models. We confirmed its extremely high potency in bacterial and cultured cell systems: its minimum inhibitory

concentration was as low as 0.01 μg · mL^{-1}, or less than 0.1 nM [24,25]. One problem, however, was its very rapid clearance in vivo by both proteolytic degradation and renal excretion.

We later found that this NCS, a small protein of 12 kDa, was being absorbed from the urinary bladder and returned to the blood circulation (by a vesicorenal recirculation mechanism) [26]. Also, high accumulation in the urinary bladder supported its use for bladder cancer. Furthermore, intravenous administration by either a bolus or continuous infusion and by subcutaneous administration led to great differences in plasma drug concentration depending on the routes of administration.

The pharmacokinetics of NCS or proteins in general were still at early stage of pharmacological sciences, and remained to be developed at that time. We later studied its pharmacokinetics and pharmacodynamics in more detail. On the basis of these data, we constructed computer-simulated pharmacokinetic compartment models for slow infusion of NCS via the carotid artery for use in therapy for brain tumors (two-compartment model) and leukemia (one-compartment model) [27,28]. These models were build based on the extremely short half-life in blood and the rapid urinary excretion. Inasmuch as a very low concentration of NCS was infused into the carotid artery for brain cancer, NCS in blood returning to the general circulation would be diluted to subtoxic levels, with concomitant proteolytic degradation help lowering its concentration below nontoxic levels. Later findings also showed that the enhanced permeability and retention (EPR) effect functions in brain cancer, because of the lack of the blood-brain barrier at the tumor site. However, this clinical strategy, based on this unique pharmacokinetic characteristic of NCS, was not fully utilized for brain tumors, except for 19 cases of glioblastoma, for which we obtained good results (unpublished). The reason for this situation was related to economics, as discussed later.

Chapter 5

SMANCS IN CLINICS AND BEYOND: THE DISCOVERY OF THE EPR EFFECT

The drawbacks of NCS, i.e., rapid renal clearance and proteolytic degradation, were overcome by conjugation of NCS with SMA polymer [17-21,29-31]. Also, the antigenicity and immunogenicity against native NCS was almost fully eliminated [20,31] like pegylated proteins. In contrast to the immunosuppression that many anticancer agents are known to cause, SMANCS stimulated natural killer (NK) cells, T cells, and macrophages, and induced interferon owing to its SMA component [32-34]. Newly introduced styrene moiety of SMANCS and SMA also confer the albumin binding property [35], thus albumin masks SMA-conjugates such as SMANCS, and thus they became more biocompatible [35]. SMANCS thus became advantageous to NCS for various reasons.

Although the biological, cytotoxic, and DNA inhibitory activities of SMANCS in vitro were almost equal to those of NCS, in that the molecular size was only 25% larger: 12 kDa versus 16 kDa, SMANCS was markedly different from the parental NCS, particularly in its hydrophobic nature and albumin-binding property. Binding to albumin would cause the M_r of SMANCS to become 90 kDa, so the apparent molecular size of SMANCS in blood plasma would exceed the molecular threshold of renal clearance, and SMANCS would not readily be cleared from the blood like NCS [27,28,31,36]. However, SMANCS leaking out of blood vessels would be cleared via the lymphatic system, as described later, and would thus exhibit lymphotropic behavior.

While we investigated these basic aspects, my colleague, Dr. Toshimitsu Konno of the First Department of Surgery of Kumamoto University Hospital, was trying to treat hepatic and biliary tumors by direct arterial injection via

laparotomy and ligation of the hepatic artery. He asked me whether SMANCS would dissolve in Lipiodol, a lipid contrast agent. If it would, SMANCS would be an ideal candidate drug for cancer treatment by arterial injection thereby he would inject SMANCS with Lipiodol. As that time, no anticancer agents were available that were suitable for use with Lipiodol. My answer was yes, it did dissolve in Lipiodol, so we prepared the SMANCS/Lipiodol solution. The next day, Dr. Konno injected this solution into the hepatic artery of rabbits with implanted VX-2 experimental tumors in the liver. When SMANCS/Lipiodol (1.0 mg $\cdot$ mL^{-1}) only 0.1 mL was injected arterially, we recognized its presence in the tumors using a soft X-ray system in the resected liver specimens, even without arterial ligation [37]. Furthermore, no laparotomy was needed, because SMANCS/Lipiodol can be administered by the Seldinger method via the femoral artery. Upon arterial injection via the hepatic artery, SMANCS/Lipiodol could spontaneously penetrated the tumor blood vessels into the interstitial tissue of tumor in a remarkable manner, which was later interpreted as the EPR effect. This finding was quickly applied to clinical settings [38-41].