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Recent developments in biomedicine fields for laser induced breakdown spectroscopy

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ABSTRACT

Laser induced breakdown spectroscopy (LIBS) can be used to determine solid, liquid, colloidal, and biological samples. It is a promising technique for analysis and characterization of the composition of a broad variety of objects. This review describes in brief the basic principles and technological aspects of LIBS, and the most recent progress of the various applications of this technique in biomedicine fields will be reviewed in detail, including bio-aerosols detection and identification, tissue analysis, mineral analysis in human body, and detection of zinc in human skin. Finally new approaches and the prospects in biomedicine fields of LIBS technique are described.

Keywords: Biomedicine, LIBS, Elemental analysis, Atomic emission spectroscopy.

1. INTRODUCTION

There are at least about 40 chemical elements in the living organisms of a human body. These elements can be grouped into three groups: The major group comprising H, C, N, O (~96.6%); The trace elements group comprising Na, Mg, P, S, Cl, K, Ca, Fe, Mn, Co, Zn and Ni (< 5%); The minor group of trace elements including V, Mo, Li, F, Si, As, Br, Sn, I and Ba (0.001%) . Studies about the possible correlation between some elements and disease are often among the medicine experts’ and biologists’ interesting. Different techniques are practiced to investigate the correlation between the consumption of certain elements and certain types of disease, including complementary DNA microarrays and serial analysis of gene expression [1]; matrix-assisted laser desorption ionization mass spectroscopy [2, 3] and surface enhanced laser desorption ionization mass spectroscopy [4]; x-ray fluorescence and proton-induced x-ray fluorescence [5, 6]. All these techniques have the common disadvantages of being time-consuming, expensive, and requiring a relatively complicated sample preparation.

This review describes a modern analytical technique based on emission of electromagnetic radiation produced after excitation of atoms, ions or molecules, which has been named Laser Induced Breakdown Spectroscopy (LIBS).

LIBS technique is a useful method for determining the elemental composition of various solids, liquids and gases. With many advantages as described in Table 1, numerous experimental as well as theoretical investigation results are found in literature as well as several review papers [7, 8, 9, 10] and three recent text books [11, 12, 13] have been published. However, only a few works related to analysis of biomedicine samples by LIBS have been reported so far. Detection of biomedical samples has become urgent because of the threats of biological warfare and epidemic spread.

Table 1. Advantages of LIBS.

<table>
<thead>
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<th>Advantages</th>
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<tr>
<td>1. The need for little or no sample preparation. The result is increased throughput, greater convenience and fewer opportunities for contamination to occur.</td>
</tr>
<tr>
<td>2. Versatile sampling for all media, including solids, gases or liquids (also conducting and non-conducting materials).</td>
</tr>
<tr>
<td>3. Very small amounts of sample (0.1 µg to 1 mg) are vaporized, therefore LIBS can be considered as quasi non-destructive.</td>
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<td>4. Permits analysis of extremely hard materials that are difficult to digest or dissolve (e.g., ceramics, glasses and superconductors).</td>
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<td>5. Analysis in micro-regions offers a spatial resolving power of about 1-100 µm.</td>
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<tr>
<td>6. Multiple elements can be analyzed simultaneously.</td>
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<tr>
<td>7. Potential for direct detection of aerosols (a solid or liquid particle in a gaseous medium) or ambient air.</td>
</tr>
<tr>
<td>8. The analysis is simple and rapid (ablation and excitation processes are carried out in a single step).</td>
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2. BASIC PRINCIPLES OF LIBS

Shortly after the laser operation was first reported in a ruby crystal in 1960, Brech and Cross [14] demonstrated the first useful laser-induced plasma on a surface. This was the “birth” of the LIBS technique and in subsequent years significant milestones were made in the development of this method.

LIBS is one method of atomic emission spectroscopy, can determine the elemental composition of a sample (solid, liquid, or gas). In LIBS, a high-power focused laser pulse focuses in or on a sample, form the vaporizing and exciting plasma, as shown in Figure 1, the spectra emitted are used to determine the sample’s elemental constituents.

3. INSTRUMENTATION

A typical LIBS experimental setup built in our laboratory is shown in Figure 2. Pulses from a laser (typically Q-switched Nd:YAG or excimer) are focused on the sample surface using a lens and an intense broadband continuum of light is released as a result of the bremsstrahlung process during the plasma formation, and then the plasma light is collected with a second lens or, as shown in Figure 2, by an optical fiber. The light collected by either component is transported to a frequency dispersive or selective device and then detected.

Each firing of the laser produces a single LIBS measurement. The spectrum is recorded by means of an array-type detector. The detector includes photomultiplier tube (PMT), photo diode array (PDA), charge coupled device (CCD), and intensified charge coupled device (ICCD). Because ICCD can be gated down to picoseconds kinetic measurements, and have excellent signal-to-noise ratio, nowadays ICCD become the popular detector of LIBS experiments. Depending on the application, time-resolution of the spark may improve the signal-to-noise ratio or discriminate against interference from continuum, line, or molecular band spectra.

Commercial LIBS instrument has come forth in recent years, and continued study and research into improving the detection limits, precision, and accuracy is required.

![Figure 1. LIBS spectrum emitted from atoms](image1)

![Figure 2. Typical LIBS experimental instrumentation](image2)
4. LIBS IN BIOMEDICINE SCIENCE

4.1. Analysis of Biological Aerosols

The analysis of microscopic particles, aerosols, and cells has received increased interest in recent years, especially bio-aerosols (bacteria, fungi, viruses, pollen) have attracted wide attention because they are found nearly everywhere and of the threats of biological warfare and epidemic spreads. Inhaled minute amounts of bio-aerosols can cause disease or toxic or allergic reactions. Thus determination and monitor the presence of airborne particulates and their actual concentration is of high interest. LIBS is found to be the most convenient technique for in-situ and real-time measurement of metal species in the gaseous and aerosol phases, thus it is suitable for analysis and characterization of biological aerosols.

Time-resolved laser-induced breakdown spectroscopy (TRELIBS) is a method that has the advantages of rapid, reliable, and highly selective. Stéphane Morel et al. [15] use TRELIBS to detection and sort species. They choose six bacteria (including Bacillus globigii as a surrogate for Bacillus anthracis) and two pollens in pellet form for detection, and get the conclusion that TRELIBS exhibits a good ability to differentiate among all investigated species, whatever the culture medium, the species or the strain.

Samuels et al. [16] analyzed bacterial spores, molds, and pollens using LIBS technique with a broadband spectrometer (200-900nm). The authors analyzed each LIBS spectrum using principal-components analysis method, and found to contain adequate information to discrimination among the biomaterials. They got the result that it was possible to discriminate between the bacterial spores and the molds and pollens.

Kim and coworkers [17] examined five bacterial strains (Bacillus thuringiensis T34, Escherichia coli H11/pHT315, Bacillus subtilis 168, Bacillus megaterium QM B1551, and Bacillus megaterium PV361) using LIBS, performing measurements directly on the bacterial culture plates. The difference in bacterial strains was clearly distinguished by two-dimensional charts of the bacterial components, calcium versus phosphate. The authors noted that their experimental results demonstrate the potential of the LIBS method for rapid and precise classification of bacteria with minimum sample preparation.

Hybl et al. [18] examined some common biological agent simulants (Bacterial spore, Media/protein, Fungal/mold spores, and pollen) using spectrally broadband LIBS system. Instead of using pellets or substrate-deposited layers, homogeneous samples were aerosolized in a micro-centrifuge tube by two ways: by making use of the laser-induced shock wave or aerosolized acoustically by dispersing a dry power suspension above a loudspeaker. From the experiments they demonstrated that LIBS has significant potential as a bio-aerosol classifier and that LIBS technique is able to resolve differing elemental ratios in bio-warfare-agent simulators and in common biological and environmental interferants.

Boyain-Goitia et al. [19] analyzed single biological micro-particles (pollens of a variety of flowers) by the method of LIBS for the first time. Their experimental results demonstrated that single-laser-pulse laser-induced breakdown spectroscopy can be performed on single biological microparticles, and that many more species need to be measured to generate a suitable reference library before detection and identification can be made reliably in real time.

In recent years many researchers are focused on the detection and identification of individual bio-aerosols using LIBS. Dixon et al. [20] demonstrated the feasibility of LIBS-based single-shot analysis of mental-rich bio-aerosols(Bacillus spores). Beddows and Telle [21] discussed the prospects of real-time, in situ laser-induced breakdown spectroscopy applied for the identification and classification of bio-aerosols (including species of potential bio-hazard) within common urban aerosol mixtures. Compared laser-induced breakdown spectroscopy measurements with data from a mobile single-particle aerosol mass spectrometer (ATOFMS), they got results that data from the ATOFMS provide statistical data over an extended period of time, highlighting the variation of the background composition. Baudelet and coworkers analyzed Escherichia coli using femtosecond pulses LIBS system [22], they also compared it with the nanosecond regime [23]. Gibb et al. [24] realized size-selective sampling of Bacillus anthracis surrogate spores from realistic, common aerosol mixtures by LIBS. Diedrich and coworkers [25] analyzed four strains of Escherichia coli bacteria using LIBS with nanosecond pulses. The experimental results show that LIBS has the ability to discriminate an environmental strain from a pathogenic strain, which suggests the possibility of using LIBS as a practical diagnostic test to identify strains obtained from environmental assays.

4.2. Tissue Analysis

Cancer diagnosis and classification is extremely complicated and, for the most part, relies on subjective interpretation of biopsy material. Automated, real-time diagnostic procedures would greatly facilitate cancer diagnosis and classification. LIBS can detect the elemental constituent in both low and high atomic number elements, and can provide rapid, non-destructive tissue analysis.

For the first time Kumar et al.[26, 27] demonstrate in principle that LIBS can be used for tissue analysis, specially the ability to differentiate between malignant and normal tissue. By analysis of malignant and normal tissue from a canine hemangiosarcoma, they found distinct differences in elemental composition in two type of sample. Figure 3 is the LIBS spectrum they got from the malignant and normal tissue cells of dog liver [27]. They concluded that the line intensity ratios of different elements can be used to determine the concentration ratio of
the trace elements in the tested tissue.

4.3. Mineral Analysis in Human Body

The analysis of important minerals and potentially toxic elements within mineralized tissue (bones, teeth, dental materials) has caused much attention in recent years.

Samek and coworkers [28, 29, 30, 31, 32] performed quantitative LIBS analysis of trace element concentration in calcified tissue. They demonstrated the possibility to distinguish unequivocally between healthy and caries infected teeth, and that LIBS analysis could be implemented and used in dental drilling using lasers. Fang et al. [33] use LIBS to analyze and identify elemental constituents of urinary calculi. They measured seven different urinary stone samples, the concentrations of elements detected with their experimental system were found to be widely different in different samples. Then they concluded that LIBS technique has the potential for routine clinic applications in urological disorder diagnosis.

Corsi and coworkers [34] measured the concentration of the main minerals present in human hair using Calibration-Free Laser-Induced Breakdown Spectroscopy (CF-LIBS) and compared their results with the results obtained through a commercial analytical laboratory, and got the conclusion that CF-LIBS is a very promising technique for hair tissue mineral analysis.

4.4. Zinc Analysis in Human Skin

It is well known that trace elements in skin, such as Zn, Ca and Fe, have important roles in the regulation of cell turnover, cell metabolism, and apoptosis.

Sun et al. [35, 36] use LIBS to detect trace metals in human skin for the first time. They used LIBS to evaluate the effectiveness of barrier creams as a means of protection against the absorption of zinc ions. Their experimental results indicated that Zn was absorbed through the skin and the concentration decreased exponentially with depth into the skin and that LIBS is a useful tool for trace elemental analysis in human skin.

5. CONCLUSIONS AND FUTURE PROSPECTS

This review paper presents the most recent development of LIBS in biomedicine fields. In the past decade there has been a burst of research activities in the use of LIBS for analysis of trace elements in biomedicine matrices. From above description we can see that LIBS is an effective technology in the detection and monitoring of a wide range of elements in human body, and that LIBS technology has great potential for clinical practice. Continued improvements in instrumentation, the understanding of laser plasma, and data analysis of LIBS technique are currently an active area of research for many groups. In the future, the research will bring extensive development of LIBS in biomedicine fields.

REFERENCES


Extrinsic electromagnetic fields, low frequency (phonon) vibrations, and control of cell function: a non-linear resonance system

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ABSTRACT

Chou and Chen’s report in the 1970s suggested conformational protein adaptation (CPA) might be influenced by low frequency phonons acting as “a possible information system”. This report proposes the universal force of electromagnetism initiates the phonon system they cited as it perturbs paramagnetic/diamagnetic dampers within the protein matrix to produce a quantized low frequency phonon signal series. The signal series is iteratively processed by the protein beta subunit, the system, to position the alpha subunit, the outcome, a classic non-linear resonance system resulting in conformational protein adaptation (CPA). CPA “priming” enables a secondary ATP/redox driven power system to complete cell activity. The evolutionary appearance of these two systems reflects their hierarchy: 1) a low energy phonon driven information control circuit governed by principles of physics that, along with proteins, may have preceded planet earth, and 2), an ATP/redox power completion circuit directed by principles of chemistry that evolved in living systems 1 billion or more years after earth formed.

Keywords: Electromagnetic fields-forcer; Paramagnetic/diamagnetic oscillators-damper; Phonon resonance-signal series; Protein iteration-system; Conformational adaptation-outcome

1. INTRODUCTION

Following the “big bang” and earlier formation of paramagnetic hydrogen, star collapse largely completed the atomic chart with three forms of electromagnetic responsive elements, i.e. paramagnetic, diamagnetic, and ferromagnetic; later, atmospheric damping of electromagnetic discharges from lightning and solar sources evolved. Schumann Resonance (SR). As life evolved on earth the SR was the primary forcer that caused bond length oscillations in paramagnetic/diamagnetic constructs (PDCs) that proteins strategically assembled to initiate and route the resultant quantized signal series. In forcing this signal series that was native to the protein matrix, the SR controlled conformational protein adaptation (CPA) in DNA, protein enzymes, and membrane proteins. Hawking noted in A Brief History of Time, “the universal force of electromagnetism controls all biological response”, applicable here just as in Pauling’s more classical derivation (Nobel lecture, 1954). Diurnal fluctuations shared by living systems and the SR constitute epochal evidence that native phonon vibrations are shared among proteins to control cell function throughout the hierarchy of living systems. For those who would assign diurnal function to light and dark a review [48] of this atmospheric influence is suggested. This phonon system, symbiotically shared as proteins assembled, and later called a meridian, was functioning nearly 2 billion years before the first nerve network evolved in Cnidarians. “Lacking a connection to the central nervous system” may not be a valid criticism of the meridian concept.

Information Control Circuit:
The low frequency phonon control circuit is a classic non-linear resonance system that involves iterative processing of this quantized acoustic series by beta sub-units in enzymes, DNA, and membrane proteins, i.e. the system, to conformationally adapt the alpha sub-unit, the outcome. With appropriate “energy coupling” facilitated, ATP/redox power completion dynamics shuttle ions and substrate along metabolic pathways, but only after CPA enables that. When investigated as a focus of life science or clinical research, these completion dynamics should be recognized as a separate rote activity for if activated without informed conformational protein adaptation the result would be chaotic or failed cell function.

While subthreshold in themselves, appropriately damped quantized phonon energy conducts heat and sound through proteins as native low frequency (phonon) vibrations to achieve resonance with harmonics from other
strategically assembled paramagnetic/diamagnetic constructs (PDCs) within the protein thus enhancing signal intensity several magnitudes [35]. DNA, and other proteins, given their sophisticated capacity to selectively extract harmonics, use these signals in combination with other “noise”, i.e. stochastic resonance, to enhance protein function [6, 18, 44]. This selective matching indicates a sophisticated routing capacity in proteins [33, 34] which is proposed here to be a co-activity of PDCs. An area of investigative interest in this regard may be the magneto-hydrodynamic effects described by Alfven (Nobel lecture, 1970) and a recent report on Hall effects [50] in phonons. This initiating/routing phonon information system promises market disruptive technologies that could dramatically enhance homeostasis as a system undergoes ischemia-reperfusion injury or other insult [12]. Extant literature [24, 31, 38] suggests significant potential for designed electromagnetic pulsed therapies (DEPTH), the technology to replace classes of drugs and numerous surgeries within the decade.

2. HISTORY

The Flexner Report in 1910 declared the universal force of electromagnetism “irregular science” and it was purged from medical curricula in the United States forty-five years after Maxwell defined it; a similar effort in Europe was unsuccessful. 1900 and “The New Era of Science” saw electromagnetism eventually characterized as “controlling all chemical reactions, including life itself” [25]. Considering this universal force and its influence on chemical reactions, it is inexplicable that clinical, biological, and basic science interest in electromagnetism, PDCs, and their relation to cell function languished until MRI stimulated interest in the late 20th century.

3. NORMAL MODE (ELEMENTARY) PHONON VIBRATIONS

Kuo-Chen Chou developed an iconic archive on the importance of low frequency phonon vibrational activity in proteins following his original insight [17]. He considered low frequency vibrations (phonons) in proteins including DNA implicit to their biological function [13, 14, 15, 16]. In citing the “overwhelming production of phonons by native low frequency vibrations”, and, “the importance of low frequency vibrations in biologic function from the viewpoint of both thermodynamic and molecular dynamics”, he alluded to the conformational adaptation of proteins and control of cell function in response to these native low frequency vibrations as, “an intriguing concept.”

W. Ross Adey accumulated an archive [1, 2, 3] on non-linear EMF cellular effects and intrinsic communication systems between cells that he considered, “a general biologic property”. Adey’s early soliton concept [36] was later abandoned by his co-author [37]. All constituent elements and amino acids function as PDC dampers within a protein lattice where, if the EM field is appropriate, an elementary or “native” mode phonon vibration results that is compatible with individual protein design and length. Given this as the means for generating phonons, Kriegl et al. [33] report additionally suggests a routing effect exists in biological systems, which prompts the question, “Does electromagnetism influence PDCs to generate phonons and direct their routing as well?” Sophisticated phonon initiation and routing directs conformational protein adaptation to prime redox reactions [52], calcium channel activity, and bioenergetics [17]; up-regulating DNA [12, 49] and enhancing enzyme activity [55] as iterative outcomes are proposed as logical conclusions in other reports.

Curie’s magnetic moment (M) of a paramagnetic species, which represents the sum of EMF directed dipole alignment (B), and Brownian vibrational activity (T), is the energy transduced into the protein lattice with sophisticated forward and reverse options [34, 27] to remote sites [6,18, 29,44]. When an EM field is damped to create a native (elementary) vibrational activity it assures homeostatic CPA to the system experiences challenge. Johnson et al. [30] noted “unambiguous” evidence for PDC presence in Fe/enzyme models, and Ubink et al. [52] reported that PDC forces directed conformational protein changes that resulted in apposition of distant protein redox sites and ensuing reaction, redox chemistry controlled by principles of physics.

“Stochastic resonance” is the ability of proteins to guide appropriate harmonics extracted from “noise”, to sum with sub-threshold phonon harmonics, another highly evolved adaptive response to enhance sub-threshold native or elementary signal series. It is unimaginable to consider that proteins would evolve a means to generate phonons without a means to route them. If time is an essential component in non-linear events [25], it should be noted that others report such distant site activations are tightly tied to protein conformational changes [51, 52], and sequential ligand relaxation times [51]. Additionally, others [33, 42] corroborate Anfinsen’s 1972 Nobel Prize findings that protein function is highly dependent on pH to speed or slow the process. For decades “classicist” argument has refuted non-linearity in cell signaling, however the iteration of quantized phonon signal series is a non-linear process (http://www.phy.ilstu.edu/~ren/phononsims/page3.html). In combination with the other inter-dependent variables it would appear a classic linear solution is an antiquated proposal.

4. ELECTRO-POLLUTION; NON-ELEMENTARY VIBRATIONS

Based upon Curie’s Law, (M=k B/T), if the imposed EM field (B) saturates PDC dipole alignment sufficiently, which need not be complete, their “damper” ability is compromised. In such an event it would seem possible to introduce extrinsic, “non-elementary modes” to cause structural or functional failure of “the system” if incompatible with the protein lattice’s ability to absorb them. Outcomes possible when a harmonic forcer drives a sys-
tem are: 1. the system changes design, e.g. a swing angle, 2. energy output must equal energy input, 3. the system fails. As noted in Fundamentals In Physics, [26], “care must be taken not to subject a system to a strong external driving force...or the resulting oscillations may rupture it”. Item 2 suggests that when heat energy in exceeds energy out the protein may be denatured resulting in a functional failure. The non-elementary vibrations associated with an EM force capable of saturating dipole activity and nullifying damping by PDCs are proposed as such oscillations. DNA due to its size, design, and quantity of electromagnetic responsive constructs may be quintessentially vulnerable to non-elementary resonant energy.

Elementary vs non-elementary dynamics must be considered when “lumping” therapeutic and “electro-pollution” field effects since one is designed to be native to the protein and the others non-elementary energy that must follow outcomes two or three. Switching large field strengths create field gradients that strongly argue against a “more is better” approach in DEPTH applications, e.g. rTMS and treatment of depression.

The upside of non-elementary resonant vibration suggests its use to destroy infectious pathogens. HIV and other pathogens undergo vulnerable dynamic adaptations, e.g. g41 fusion protein, that promise compromised function or structure with appropriate vibrational exposure. Successful eradication of Plasmodium falciparum in Ghana after a single DEPTH treatment has been reported [22]; that report cites a different vibrational mechanism than is suggested here.

Trial and error will be necessary to find non-elementary vibrational modes necessary to degrade the function or structure of vulnerable proteins. Anecdotal observations over 25 yrs on treating infectious conditions with DEPTH, e.g. fistul, otitis, lymphangitis, suggests the originating EM field can be within therapeutic limits with outcomes that indicate a startlingly rapid functional or structural compromise of agent virulence, if not viability. Satisfied with the rapid dehiscence of pain and inflammation, one overlooked the obvious implication in permanently improved outcomes that required no additional treatment. Toxic metals and chemical toxins may substitute very different energy into PDC bonds, which then oscillate at very different output modes than the original construct to create a “non-elementary” response to an otherwise normal EM force, perhaps the long-term lethal threat in such bond substitutions.

5. ELECTROGENOMICS

The use of designed EM pulses to up-regulate DNA received substantial support when the Columbia University group headed by Blank and Goodman reported that site-specific electromagnetic responsive elements (EMRE's) regulate DNA synthesis [11, 40]. In spite of their observation these authors propose other than paramagnetic/diamagnetic dynamics, signal series, and conformational adaptation of DNA to activate gene loci. It could be proposed that electron transit across weak hydrogen bonds between base amino acid pairs doesn’t occur “de novo”, which suggests such enthalpy is an iterative, conformational adaptive activity. This same group reported that up-regulation was increased when transduced via multiple, similar EMRE constructs vs. just one or two [40], which strongly suggests an EMF/PDC/phonon iteration effect as the first order mechanism. [6, 18, 44]

6. CALCIUM AND Ca++ CHANNEL ACTIVITY

Eichwald and Walleczech [21] reported the biological-functional status of treated tissue can result in EMF stimulation, inhibition, or no effect, which they attributed to “activation of specific EMF sensitive enzyme systems that modulate calcium entry.” Calcium is an alkaline earth metal, diamagnetic, and considered “very reactive” [56]; it and water are both diamagnetic and highly mobile in EM fields, which along cell membranes with their myriad PDCs can only be imagined. Without noting its diamagnetic nature, calcium flux in response to EM fields was reported [39], and Dihel et al. [29] demonstrated such observations were related to Ca channel activity. Davies and Norris [19] demonstrated that Ca++ dependent motility in marine diatoms, was substantially enhanced by EMFs. Using pheochromocytoma cells, Ikehara et al.[28] demonstrated EMFs inhibit increases in cytosolic [Ca++] by limiting release from intracellular stores, which was rapid (15 min) and lasted for two hours in the face of unchanged ATP levels over that period. Gibbs et al demonstrated cysteine rich secretory protein domains regulate ion channel activity, “and provide compelling evidence for a role in [Ca++] regulation...” Cysteine is proposed a classic PDC.

Vendel et al [54] reported, “Ca++ channel beta sub-units regulate trafficking and gating of voltage dependent Ca++ channel alpha subunits”, indicating beta subunits are the protein intelligence that computationally processes acoustic signal series. Using signal series to position voltage gated alpha subunits into appropriate position to achieve homeostasis is the precisely computed outcome of this “low voltage” information control circuit; given this direction, the parallel ATP driven energy system fulfills its “power circuit” role by appropriately moving substrate and solute to complete the homeostatic effort.

CPA (folding) has been demonstrated as a direct PDC effect [52], and Rosen’s [47] reference to Ca++ channel deformation from “anisotropic diamagnetic” phospholipids in the cell membrane might be expanded to incorporate many other PDCs [7], not to mention transitional metals. Vendel [53] further notes, “these domains that regulate cell surface expression and movement of Ca++ voltage gated alpha sub-units involve five domains related to, “a large family of membrane-associated guanylate kinase proteins”, conformational adaptation suggested to be a (phonon) signal series driven outcome. Mustafi et al. [43] report essentially similar findings in terms of multiple domains as part of “paramagnetic interactions with diamagnetic lanthanides, which substitute for Ca++”.

PDCs are major contributors to conformational protein
control of Ca++ channel activity, very probably Ca++ routing through the channel, and cytosolic [Ca++] release [28]. Lastly, Baureus Koch et al. [8] studied weak EMF effects on the cell membrane and found, “suitable combinations of time varying magnetic fields directly interact with Ca++ channel proteins in the cell membrane”.

7. CONCLUSION

Adey noted [3], “Today we stand at a new, far more significant frontier, and while it may be more difficult to understand, it is at the atomic level rather than the molecular that physical rather than chemical processes shape the flow of signals essential to living matter . . . one of the great revolutions in the history of biology”. Adey was an MD who spent much of his career seeking the connection between cell function and the universal force of electromagnetism. This author proposes that PDC response to electromagnetic forcers, which has been overlooked for a century, keys the sophisticated information network proteins rely upon for conformational adaptive response [1, 2, 9, 41, 45, 46, 52]. Almost as an afterthought, redox reactions and ATP complete cell response.

It is apropos that we modify EB Wilson’s 1950s quote, [4], “the key to every biologic problem must finally be sought in the cell” to that of a 2008 version, “must finally be sought in the physics of phonons, native low frequency vibrations that drive conformational protein adaptation”, the cell will follow. Phonon physics and conformational protein adaptation are central to the paradigm beyond molecular physiology. Recent articles [6, 18] report DNA’s use of noise to up-regulate gene synthesis and the sophisticated capacity of proteins to extract specific harmonics from overtones to enhance sub-threshold acoustic information. Electromagnetism both initiates and routes these vital phonon signals to control biological response; a sophisticated information system would have it no other way.

This understanding suggests overlooked avenues to control cell function, DNA up-regulation, and enzyme activity in response to ischemia-reperfusion injury and other threats that will be better served by technologies as opposed to current drug regimens and numerous surgeries.

Proteins initiated this low energy conformational adaptive system upon first evolving, two or more billion years before the appearance of a nerve network, and still further removed from a central nervous system. A step toward returning to Rachel Carson’s, “natural balance of things” and the human condition would be to enhance iterative protein activity rather than “blocking” or “inhibiting” it.

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Identification of essential language areas by combination of fMRI from different tasks using probabilistic independent component analysis

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ABSTRACT

Functional magnetic resonance imaging (fMRI) has been used to lateralize and localize language areas for pre-operative planning purposes. To identify the essential language areas from this kind of observation method, we propose an analysis strategy to combine fMRI data from two different tasks using probabilistic independent component analysis (PICA). The assumption is that the independent components separated by PICA identify the networks activated by both tasks. The results from a study of twelve normal subjects showed that a language-specific component was consistently identified, with the participating networks separated into different components. Compared with a model-based method, PICA’s ability to capture the neural networks whose temporal activity may deviate from the task timing suggests that PICA may be more appropriate for analyzing language fMRI data with complex event-related paradigms, and may be particularly helpful for patient studies. This proposed strategy has the potential to improve the correlation between fMRI and invasive techniques which can demonstrate essential areas and which remain the clinical gold standard.

Keywords: fMRI, probabilistic independent component analysis (PICA), language mapping, event-related paradigm

1. INTRODUCTION

The purpose of pre-surgical language mapping is to lateralize and localize critical language areas for neurosurgical planning when the patient’s lesion is located in or close to language areas. In addition to invasive language mapping techniques (e.g., intracarotid amytal test (IAT), and intra-operative electric cortical stimulation (ECS)), pre-operative functional magnetic resonance imaging (fMRI) based on language tasks has been used to determine the language-dominant hemisphere [1] and provide spatial relationships between brain lesions and language areas [2]. Although fMRI has the advantages of non-invasiveness, pre-operative availability, repeatability, and less time and cost, it has several shortcomings for language mapping applications [3]. First, compared to the conventional inhibition methods that are able to demonstrate essential areas, fMRI is an observation method which thus demonstrates numerous areas involved in the language tasks, but cannot demonstrate the necessity of those areas in language function. Second, language fMRI generally uses silent tasks due to the motion artifact resulting from vocalizing responses, which complicates comparisons with the clinical gold standard tests that use overt responses.

To try to address these problems, we applied a data-driven method, probabilistic independent component analysis (PICA) [4], to fMRI data from two language tasks. First, we propose an analysis strategy to examine activations during two different tasks in an effort to identify the essential language areas. Second, we investigate the performance of PICA in extracting language-related components from vocalized language fMRI data that are contaminated by motion artifact and background noise.

Independent component analysis (ICA) has been applied to fMRI data to extract statistically independent features [5, 6]. It has been shown that ICA can be used as a complementary tool to the conventional general linear model (GLM) method in improving the sensitivity and specificity of fMRI language mapping [7]. PICA is an extension of the classical noise-free ICA model [5], assuming that the data are confounded by additive Gaussian noise [4]. It was proposed to address the overfitting problem and make the statistical significance testing feasible for the analysis of fMRI data. PICA has been applied in several studies, including the investigation of the neural dynamics of default-mode networks and event segmentation in music [8-11].
2.1. Subjects and Image Acquisition

The protocol was approved by the Partner’s Institutional Review Board. Twelve right-handed native English speaking healthy subjects participated (7 men, 5 women, mean age = 30.6 ± 6.5 years, range 20-43 years). One right-handed patient with focal cortical displasia (female, 35 years old) was also studied. All subjects provided written informed consent.

MR images were obtained using a 3.0 Tesla scanner (Signa scanner, GE Medical System, Milwaukee, WI, USA). A single-shot gradient-echo echo-planar imaging (EPI) was used to acquire blood-oxygen-level dependent (BOLD) functional images (TR = 1000 ms, TE = 29 ms, flip angle = 68°, FOV = 24 cm, dimension = 64 x 64 x 16, slice gap = 0 mm, voxel size = 3.75 x 3.75 x 5 mm³) using a quadrature head coil. In each image volume, 16 axial slices were acquired using ascending interleaved scanning sequence. A volumetric T1-weighted magnetization prepared rapid gradient echo (MPRAGE) acquisition (dimension = 256 x 256 x 124, voxel size = 1 x 1 x 1.3 mm³) was performed to provide a high-resolution anatomic reference frame for subsequent overlay of functional activation maps.

The patient’s functional images were acquired using different parameters (TR = 2000 ms, TE = 40 ms, flip angle = 90°, FOV = 24 cm, dimension = 128 x 128 x 28, slice gap = 0 mm, voxel size = 1.88 x 1.88 x 4 mm³, ascending interleaved scanning sequence). High-resolution T2-weighted gradient-echo MR images (dimension = 512 x 512 x 91, voxel size = 0.5 x 0.5 x 1.5 mm³) were acquired to provide background structural images for the patient’s activation maps.

2.2. Behavioral Paradigm

Subjects performed two language tasks with vocalized responses: antonym-generation (AG), and noun categorization (NC). In the AG task, subjects were asked to speak the antonym of a word stimulus presented visually through MR-compatible video goggles (Resonance Technology, Los Angeles, CA, USA). In the NC task, subjects were asked to state whether the word stimulus referred to either a living (“alive”, e.g., a dog) or non-living (“not alive”, e.g., a chair) object. Subject vocalizations were transmitted by an intercom system (Avotec Inc., Stuart, FL, USA) to an investigator in the MRI scanner control room who counted the number of incorrect or omitted responses in order to verify satisfactory task performance. Subjects were instructed to verbalize responses with minimal movement of their head, jaw, or lips. During the time period between visual stimuli, subjects were asked to relax and look at a cross-hair shown in the center of the visual field.

The language tasks were implemented as a rapid-presentation, event-related fMRI paradigm with a jittered inter-stimulus-interval (ISI = 8.3 ± 5.1 sec), lasting 7 min 20 sec (including a 10-sec pre-stimulus period acquired to allow stabilization of the BOLD signal, excluded from analysis). A total of 50 word stimuli were delivered during each task, and each word was shown for 2 sec. The fMRI paradigms of the patient study lasted 5 min 20 sec (including a 10-sec pre-stimulus period), delivering 34 words in the AG task and 39 words in the NC task). The order and exact timing for delivery of word stimuli was based on a stochastic design intended to maximize the statistical significance of the fMRI paradigm, and minimize subject’s expectation and habituation effects. Stimuli event scheduling was performed using the Optseq2 software package (NMR Center, Massachusetts General Hospital, MA, USA). Stimulus paradigms were implemented using Presentation software package (Version 9.70, Neurobehavioral Systems Inc., Davis, CA, USA).

3. METHODS

3.1. Concatenation of Two Tasks and Data Pre-processing

FMRI data from two tasks were concatenated in time by putting the NC task data at the end of the AG task data (Figure 1). Thus the total data set of each subject was 860 volumes (430 volumes for each task). Then we used the Statistical Parametric Mapping software package (SPM2, Wellcome Department of Cognitive Neurology, London, UK) to perform motion correction by realigning the fMRI images to the first functional image.

The data were then sent to the Multivariate Exploratory Linear Optimized Decomposition into Independent Components (MELODIC, Version 3.05) module of FMRIB’s Software Library (FSL, Version 4.0, Oxford Center for Functional Magnetic Resonance Imaging of the Brain, University of Oxford, Oxford, UK) for PICA analysis [4]. Before the PICA procedure, the following steps were applied to the input data file: masking of non-brain voxels by an intensity thresholding at 10%; high-pass temporal filtering to remove low-frequency drifts with cut-off period of 128 sec; and voxel-wise de-meaning and variance normalization of the data.

3.2. Probabilistic ICA of fMRI Data

We applied the PICA technique proposed by Beckmann and Smith [4] to analyze the concatenated fMRI data. The fMRI signal (X) is assumed to be generated from a linear mixing process of the independent non-Gaussian sources (S) by a mixing matrix (A), and corrupted by additive Gaussian noise (η):

\[ X = AS + \eta. \] (1)

In the PICA model (1), X is a \( p \times n \) matrix denoting \( p \) volumes (\( p = 860 \) volumes for this study) of \( n \) voxels fMRI data, \( S \) is a \( q \times n \) matrix denoting \( q \) non-Gaussian sources (i.e., independent components, ICs), and \( A \) is a \( p \times q \) mixing matrix.

First, the number of ICs (\( q \)) was estimated using the Laplace approximation to the Bayesian evidence of the model order [4]. There were 95 ~ 166 components estimated for each subject (mean ± STD = 122 ± 20, across...
Figure 1. Concatenation of two tasks fMRI data. Red bars: word stimuli onsets.

Figure 2. Spatial maps of (A) "language COI", and (B) "motor COI" of an example subject estimated by the PICA analysis (posterior probability thresholded at $p > 0.5$).

The fMRI data were then reduced to $q$ dimensions by principal component analysis (PCA) and decomposed into $q$ spatially independent components by the fastICA algorithm [12]. A de-mixing matrix $W$ was found to generate:

$$\hat{\mathbf{S}} = WX,$$

where $\hat{\mathbf{S}}$ is a good approximation to the sources.

Then the estimated component maps were divided by the standard deviation of the Gaussian noise to generate z-score maps, and sequentially thresholded at a posterior probability $p > 0.5$, by fitting a Gaussian/Gamma mixture model to the histogram of intensity values [4].

3.3. Identification of Components of Interest

We used a two-step process to identify the components of interest (COIs) from the PICA output of each subject’s data. First, the power spectrum density of each component’s time course was estimated by periodogram spectral estimation. Then the average power was calculated for frequency less than 0.1 Hz, and ranked in descending order. This frequency range was selected based on the power spectrum of the expected hemodynamic response function (HRF), which was in low-frequency range (< 0.1 Hz). Next, the components’ spatial maps were visually inspected in the order determined by the previous step to identify the components with activation in the putative language areas, and other areas of interest.

3.4. GLM Analysis of fMRI Data

For comparison purposes the pre-processed data were submitted to SPM2 for conventional GLM analysis. The basis function consisted of the canonical HRF model with temporal and dispersion derivatives. Run-specific responses were modeled in an event-related design [13] by convolving a series of Dirac’s delta function, each representing a stimulus event onset, with the basis function. After GLM, the $t$ maps were fitted to a Gaussian/Gamma mixture model and thresholded at a posterior probability $p > 0.5$ in order to be comparable with the PICA results.

4. RESULTS
Figure 4. Temporal correlation coefficients between the identified COIs’ time courses and the expected HRF model for each subject (red bars: language COIs; blue bars: motor COIs).

Figure 5. Spatial maps of GLM analysis of the same example subject as in Fig. 2 (posterior probability thresholded at $p > 0.5$).

4.1. Spatial Maps of Identified Language and Motor COIs

For each subject, among an average of 122 components estimated by PICA, one component was consistently identified with activations in the left frontal and temporal lobes, primarily in the putative language areas (inferior frontal gyrus, superior temporal gyrus, and supramarginal gyrus), and pre-motor area (PMA, middle frontal gyrus). This component was named “language COI”. Figure 2A shows the spatial maps of the language COI of an example subject (activations in the putative language areas and PMA are highlighted by red circles). Another component was identified with bilateral activations mainly in the primary face motor areas (precentral gyrus), which was named “motor COI”. Figure 2B shows the spatial maps of the motor COI of the same subject.

The ranking results of the language and motor COIs based on the average power of their time courses are listed in Table 1 for each subject. It demonstrated that these COIs were ranked within the first 15 ICs (except for one subject (#10), whose language COI ranked the 23rd, and motor COI ranked the 25th).

4.2. Time Courses of Identified COIs

Figure 3 shows the time courses of the language (red) and motor (blue) COIs averaged across all subjects. The expected HRF model is also shown (green).

Figure 4 shows the results of temporal correlation analysis between the identified COIs’ time courses and the expected HRF mode for each subject. It demonstrated that the time courses of the motor COIs of 7 subjects correlated more closely with the expected HRF than that of the language COIs. The correlation coefficients are $0.30 \pm 0.17$ (mean $\pm$ STD, across subjects) for the language COIs, and $0.38 \pm 0.25$ for the motor COIs.

4.3. Comparison of PICA and GLM Results

Figure 5 shows the spatial maps of the GLM results of the same example subject. Compared with the PICA maps, the GLM maps identified activation patterns very similar to that of the motor COI (Figure 2B), with bilateral activations mainly in the primary face motor areas. The GLM maps showed weak activations in the putative language areas.

4.4. Results of Patient Data
The spatial maps of the language and motor COIs resulting from PICA analysis of the patient data are shown in Figure 6. The “language COI” (Figure 6A) showed significant activations in the left inferior frontal gyrus and left PMA. The “motor COI” (Figure 6B) shows bilateral activations in the primary face motor areas. Temporal correlation analysis indicated that the language COI correlated poorly with the task timing \( (r = 0.15, p < 0.01) \), while the motor COI correlated highly with the task timing \( (r = 0.45, p < 0.0001) \). Using the same pre-processed data, GLM generated maps (not shown) revealed activations mainly in the bilateral motor areas, but almost no activations in the putative language areas.

Figure 7 shows two noise components indicating signals from the edge of the brain (due to motion artifact, Figure 7A) and the ventricle system (Figure 7B).

5. DISCUSSION

In this study, we propose a new analysis strategy using PICA and combining fMRI data from different language tasks to better identify the essential language areas for pre-operative language mapping. A component was consistently identified in the putative language areas, suggesting that the underlying system was essential to both tasks. Compared with the conventional GLM method, PICA indicated better performance in extracting language activations, and separating noise into different components.

It is clinically useful to demonstrate brain regions critical for language function so that they may be avoided during resection [3]. Whereas, clinical tests which block neural activity (IAT and ECS) are able to demonstrate the necessity of particular areas for language function, fMRI maps may include non-language-specific areas that participate in the task [7]. To try to demonstrate language-specific areas, we concatenated the fMRI data from two different tasks in time, and applied PICA to estimate the spatially independent components from the data. The underlying assumption is that the networks revealed by PICA are activated by both tasks, which are more likely to be essential language areas. The results of all subjects consistently identified a network in the putative language areas and the ipsilateral PMA. Another network was found in the bilateral face motor areas, indicating the participation of these areas in overt language production.

The temporal correlation analysis on the time courses of the “language COIs” showed a relatively low correlation with the expected HRF model, while the “motor COIs” correlated more closely with the HRF model. This indicated that the temporal profile of the language activation may differ from the task timing, while the motor activation followed the task very well. Since the GLM method is based on the expected HRF model, and therefore able to identify voxels whose time courses correlate highly with the paradigm, this may explain the observation that the GLM identified very similar activation patterns to the “motor COIs”, but did not show significant activations in the language areas. PICA’s ability to capture the neural networks whose temporal activities may deviate from the time course of the paradigm suggests that the data-driven method may be well-suited to analysis of complex event-related language fMRI. The data-driven method could be particularly useful in analyzing patient data, since the lesions adjacent to the language areas may result in alterations in the BOLD response [14], and patients may have difficulty with task performance causing altered timing of the cognitive process relative to the model.

To identify components of interest from a large number of separated components remains a practical challenge for the ICA technique, and methods have been proposed based on spatial, temporal, and spectral criteria [5-7, 15]. In this study, we ranked the ICs based on their time courses’ average power in the frequency range of less than 0.1 Hz. This frequency range was chosen based on the power spectrum of the expected HRF model. The language and motor COIs were among the highly ranked ICs, which confirmed the effectiveness of this selection criterion. Among other highly ranked ICs were default-mode networks as shown in [8], and low frequency head motion artifact.

Vocalized event-related language paradigms offer an advantage of more closely simulating natural language performance. However, the motion artifact resulting from vocalizing responses may lead to contamination in the statistical maps. PICA has the ability to separate signals from motion artifact, noise, and physiological effects, into different components, and therefore may be particularly applicable to vocalized language fMRI data. This proposed combination strategy can be extended to fMRI data from multiple tasks. To improve the effectiveness of this strategy, the different tasks should be
selected in an optimal way. In this study, the AG task focused on both the receptive and expressive aspects of language function, and the NC task was more involved in the receptive aspect. Future work will be directed at the optimal combination of task paradigms, as well as validation of this approach against invasive testing in patients.

6. CONCLUSION

We propose a new analysis strategy to identify essential language areas by combining fMRI data from two different tasks. We applied a data-driven method, PICA, based on the assumption that the separated spatially independent networks were activated by both tasks. The results show that using this approach, the language component was consistently identified and separated from the participating networks. This approach compares favorably with GLM for complex event-related language paradigms, and may be particularly helpful for patient studies for pre-operative language mapping.

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In vitro and in vivo cell tracking of chondrocytes of different origin by fluorescent PKH 26 and CMFDA

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ABSTRACT

Tissue engineering techniques for cartilage repair to heal defects in joint surfaces is a clinical practice. Harvested autologous chondrocytes are expanded in culture and delivered in a suitable carrier medium back into the patient’s joint defect. The defect is then subsequently filled by new cartilage. Whether the cells in the repair tissue originate from the engineered tissue of the host or are derived from the surrounding original cartilage remains a relevant question for the applied therapy. To answer this several methods exist to track cells, such as transfection of cells with LacZ carrying viruses, radio labeling with 111 In or 51 Cr or fluorescent labeling with FDA. However, these techniques have drawbacks such as they may influence cellular properties, are radioactive or quickly lose their tracking ability. New fluorescent probes are easier to handle and do not to interfere with cells.

PKH 26® is a relatively new cell-labeling agent, but few data exist on the application of this dye in chondrocytes in vitro and in vivo. 5-chloromethylfluorescein diacetate - CMFDA (“cell tracker green”) is an established fluorescent probe for imaging the dynamic processes of cell proliferation in vitro and in vivo. Likewise, several studies exist on different cell types. However, little data are available for chondrocytes.

The first aim of this study was to evaluate qualitative differences in fluorescence pattern after labeling of articular, auricular and costal chondrocytes. Secondly, we evaluated the influence of labeling with CMFDA on cellular adhesion properties. The third aim was to compare the duration of cell labeling of chondrocytes of different origin with established CMFDA as standard and PKH 26® for 3 cell generations in vitro and 12 weeks in vivo. We show that chondrocytes from different origin can be labeled effectively with both PKH 26® and CMFDA. The PKH 26® labeled articular chondrocytes maintained fluorescence longer than CMFDA in vitro and in vivo. A higher percentage of articular chondrocytes remained stained at 63 days than auricular or costal chondrocytes.

Keywords: Cell tracking, cell generations, integration, fluorescence, chondrocytes of different origin, CMFDA, PKH 26, tissue engineering

1. INTRODUCTION

Applied tissue-engineering techniques are clinical reality in joint cartilage replacement and defect filling. The clinician harvests chondrocytes from the patient’s joint surface of a non-weight bearing area. After expansion in culture the cells are re-implanted into the patient into an existing defect using a suitable carrier for the cells. The relevant question to justify the technique remains weather the defect surrounding chondrocytes are migrating into the lesion or if the new tissue originates from the implanted cells. To answer this durable and stable methods for cell staining are essential in long term follow up studies of transplanted cells in order to allow identification of the origin of the daughter cells. Several features are necessary. The marker should be 1: detectable after a prolonged period of time, 2: be easy to handle, and 3: should not interfere with immunocytochemical staining to characterize the fate and differentiation of grafted cells in the host tissue, such as cartilage.

Different methods have been established to identify transplanted cells in host tissue such as bromodesoxyuridine – BrdU [7, 15] labeling of cells with [3H] thymidine [2, 14, 15] or transfection with the LacZ gene or beta – galactosidase [6, 12, 22] but each techniques has some method specific disadvantages, such as involved radioactivity or special security measures are needed when handling viruses. Newer labeling techniques use chemical integration of fluorescent dyes into cellular structures such as organs or membranes. PKH 26® has been described as a fluorescent dye integrating irreversibly into the cellular membrane of living cells, and its labeling efficiency is more than 90% for many different cell types [9, 18, 20, 21, 23]. However, little data is available
on its effectiveness with chondrocytes. Chondrocytes have a slower metabolism and replication rate compared to liver cells or mesenchymal stem cells [10, 11]. In addition, chondrocytes produce an extracellular matrix that encapsulates them and that is different from other matrices produced by e.g. osteocytes or keratinocytes. For these reasons cell-tracking dyes mentioned before might not be the best choice for chondrocytes. Several authors have recommended the use of CMFDA for labeling different cell types for long-term studies, which is easy to handle. It integrates into the cytoplasm of viable cells, independent from the cell cycle, and has been used in bone marrow derived stem cells [8] keratinocytes, myocytes and osteocytes [3], lymphocytes and U937 cells [4]. Despite these advantages, the duration of the labeling is not known and only few data are available on its use with chondrocytes. In a previous in vivo experiment, we used PKH 26® to label transplanted auricular and articular chondrocytes and osteocytes [5], lymphocytes and U937 cells [4], bone marrow derived stem cells [8] keratinocytes, myocytes and osteocytes [3], lymphocytes and U937 cells [4]. Despite these advantages, the duration of the labeling is not known and only few data are available on its use with chondrocytes. In a previous in vivo experiment, we used PKH 26® to label transplanted auricular and articular chondrocytes and osteocytes [5], lymphocytes and U937 cells [4].

We evaluated the differences in PKH 26® and CMFDA durability of cell labeling in vitro for 9 weeks for 3 daughter cell generations and in vivo for 12 weeks in a long-term study. Also, differences in labeling of three different kinds of chondrocytes and the influence of the marker on cell replication rate were examined.

2. MATERIAL AND METHODS

2.1. Chondrocyte Preparation

Under sterile operating conditions articular, auricular and costal cartilage from 6-month-old Yorkshire swine were harvested. The cartilage was excised, rinsed in phosphate-buffered saline (PBS) with 2% antibiotics, and minced into 1-mm³ pieces. The cartilage was digested at 37°C in a 5% CO₂ incubator for 12-18 hours in Ham's F-12 media with Glutamax-1 (Gibco/BRL, Life Technologies, Grand Island, NY) containing 0.1% of collagenase Type 2 (Worthington Biochemical, Freehold, NJ) and 1% antibiotics. The solutions were then passed through a 100-µm filter to remove undigested particles. The cell suspension of isolated chondrocytes was centrifuged at 1500 rpm for 10 minutes, washed 3 times with PBS containing 1% antibiotic/antimycotics (10,000 units penicillin, 10mg streptomycin and 25 µg amphotericin B, Sigma Chemical Co., St Louis, MO) and cell viability assessed using trypan blue exclusion. Only cells with a viability of >90% were used in this study. Cell counting was done by a hemocytometer to the nearest 1x10⁶ cells per ml.

2.2. Chondrocyte Labeling with PKH 26®

PKH 26® (Sigma Chemical Co., St Louis, MO): PKH 26® is a lipophilic dye with aliphatic tails that binds irreversibly into the lipid regions of the cell membrane. The amount of dye is then partitioned equally between daughter cells during mitosis and therefore decreases by half at each cell division.

All steps were performed at 25°C, according to the manual of the producer. The cells were placed in a conical polypropylene tube (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at concentrations of 2x10⁶ cells per ml and were washed once with serum free medium. The chondrocytes were centrifuged for 5 minutes, the supernatant aspirated and re-suspended in 1ml of Diluent C. A stock solution of PKH 26® of 4x10⁻⁶ M was prepared immediately before staining, added to the Diluent C/chondrocyte suspension, suspended and incubated at 25°C for 5 minutes. The tube was intermittently inverted to assure mixing. After adding 2ml of FBS (Sigma Chemical Co., St. Louis, MO) the cells were incubated for 1 minute and then the suspension was diluted with 4ml of complete medium, consistent of Ham’s F12 medium with 10% FBS, 1% Glutamine (Mediatech Cellgro, VA), 50mg L-Ascorbic acid (Sigma Chemical Co., St. Louis, MO) and 1% antibiotics/antimycotics. The suspension was centrifuged for 10 min at 25°C, the supernatant aspirated and the cells were transferred to a new tube for further 3 times of washing with 10ml of complete medium.

2.3. Chondrocyte Labeling with CMFDA

5 chloromethylfluorescein diacetate - CMFDA or “Cell tracker green” (Molecular Probes Inc., Eugene, OR): CMFDA passes through the cell membranes into the cell, where the chloromethyl group reacts with intracellular thiols, leading to a cell-impermeant fluorescent dye. CMFDA stained cells have been found to fluoresce brightly for at least 72 hours after incubation in fresh medium at 37°C and last through at least four cell divisions.

CMFDA was warmed to room temperature and dissolved with anhydrous dimethylsulfoxide (DMSO) to a final concentration of 10 mM. This stock solution was diluted to a final working concentration of 5 and 10 µM in serum free medium. We found the best labeling concentration to be 10 µM. All cells were spun in a centrifuge to obtain a pellet and the supernatant was aspirated and re-suspended in previously warmed probe containing medium. The cell medium suspension was then incubated for 45 minutes under growth conditions. Thereafter the cells were centrifuged once more, re-suspended in fresh pre-warmed medium and incubated for 30 minutes. The cells were then washed again.

2.4. Seeding and Sample Preparation

Successful labeling of the cells was confirmed by examination under a fluorescence microscope. Unlabeled controls did not fluoresce when evaluated under fluorescent microscope.

Cells were obtained for culturing and plating on 25cm² flasks (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). We obtained auricular, articular and costal chondrocytes from native cartilage by collagenase (0.1%) diges-
tion. 400,000 cells were labeled, the rest served as unlabeled controls. The cell density in the flasks was 16,000 cells per cm². From each chondrocyte source at least 3 flasks were prepared. Media was changed every other day for the plated and seeded cells. Photos were taken every 7 days from the flasks. All pictures were digitized to evaluate number of fluorescent cells for each cell type. This was performed with MetaMorph (Expansion Programs International, Inc. (Thunderstone), CA, U.S.A.), by 3 times counting 250 cells at each time point for each cell type, using random counting grids of the fluorescent microscope at predetermined schedule every 7 days until 63 days (9 weeks). Percentage was calculated and the mean value determined for each time point of observation (Table 1). The chondrocytes were passaged when confluent, until the third passage was reached at 16,000 cells/cm², using 0.25% Trypsin. The counted cells at the fixed counting schedule every 7 days were then compared to the cell number achieved at each passage.

For the in vivo part of the experiment chondrocytes of each source were labeled with PKH 26® and CMFDA. These cells and unlabeled controls were encapsulated in fibrin glue to a final concentration of 40 million cells per ml. Immediately after the gelation of fibrin glue the constructs of 0.2 ml volume were inserted into the subcutaneous pouch of a nude mouse. The constructs were harvested after 3 and 12 weeks time. The constructs were examined morphologically, snap frozen in liquid nitrogen and sent for histological examination. Frozen sections of four micrometers were taken and examined under a fluorescent microscope. The percentage of fluorescent cell number in the tissue was determined by the MetaMorph (Expansion Programs International, Inc. (Thunderstone), CA, U.S.A.) program analysis.

3. Results

3.1. Cultured Chondrocytes in Flasks

Chondrocytes in culture were monitored until stain was no longer observed. PKH 26® labeled chondrocytes reflected brightly under the fluorescent microscope. Labeled cells with PKH 26® of all three sources were clearly separable from the background noise for the first 48 days. The chondrocytes were followed up until day 63, when fluorescence was no longer observed (Tables 1, 2). Weekly observation of the labeling of the chondrocytes showed less labeled chondrocytes after 14 days of culture. However, cells were still clearly distinguishable from background noise. After 28 days, fluorescence was less clear on fewer cells in each flask. Until day 63 the number of fluorescent chondrocytes decreased gradually. Only single cells were fluorescent, the articular chondrocytes revealed the clearest fluorescence and the highest number of labeled cells from day 49 (Tables 1, 2, Figure 1).

Chondrocytes labeled with CMFDA showed fluorescence only until day 14. After that all three types of the chondrocytes did not reveal any further fluorescence capacity. CMFDA labeled auricular chondrocytes and controls were confluent after 12 days. Both, PKH 26® labeled auricular chondrocytes and auricular controls were passage first after 10 days of culturing. Each flask contained 1 million cells, i.e. 2.5 times the original cell number. More than 79% of the cells were fluorescent. At the second passage after 15 days in culture, more than 75% of the chondrocytes were labeled. The flasks contained 1 million cells, equaling 2.5 times the plated cell number. The third passage of PKH 26® labeled auricular chondrocytes was at 25 days, showing more than 50% of the cells labeled; controls were confluent after 29 days of culture. Labeled and unlabeled costal chondrocytes were confluent after 16 days. Flasks contained 1.5 million cells, 4 times the cell number of original plated cells. More than 71% of the labeled cells were fluorescent. At 28 days at the second passage, more than 52% costal chondrocytes were fluorescent. The flasks contained 2.5 times the original plated cell number. The third passage was performed after 42 days with 43% labeled chondrocytes. We counted 1 million stained and 1 million control chondrocytes in each flask equaling a 2.5 times passed number of costal chondrocytes. PKH 26® labeled articular chondrocytes and controls were first passaged after 20 days. More than 70% of the chondrocytes were labeled. Flasks held 1 million chondrocytes each, which is a 2.5 times doubling rate. Articular chondrocytes were second time passaged at 35 days. 1 million chondrocytes per flask were counted, equaling 2.5 times the passaged cell number. The third passage at 42 days showed 44% of the cells fluorescent. Flasks contained 2.5 times the original plated cell number, i.e. 1 million

### Table 1. PKH 26® labeled chondrocytes – articular cells showed the longest lasting staining.

<table>
<thead>
<tr>
<th>Day</th>
<th>Articular Cells</th>
<th>Auricular Cells</th>
<th>Costal Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>87.9%</td>
<td>91.1%</td>
<td>84.7%</td>
</tr>
<tr>
<td>14</td>
<td>73.4%</td>
<td>70.2%</td>
<td>76.7%</td>
</tr>
<tr>
<td>21</td>
<td>70.4%</td>
<td>73.2%</td>
<td>71.0%</td>
</tr>
<tr>
<td>28</td>
<td>63.2%</td>
<td>50.3%</td>
<td>52.5%</td>
</tr>
<tr>
<td>35</td>
<td>50.3%</td>
<td>47.3%</td>
<td>48.2%</td>
</tr>
<tr>
<td>42</td>
<td>44.1%</td>
<td>42.0%</td>
<td>43.3%</td>
</tr>
<tr>
<td>49</td>
<td>41.0%</td>
<td>36.2%</td>
<td>37.1%</td>
</tr>
<tr>
<td>56</td>
<td>33.1%</td>
<td>26.6%</td>
<td>24.3%</td>
</tr>
<tr>
<td>63</td>
<td>22.1%</td>
<td>8.3%</td>
<td>10.4%</td>
</tr>
</tbody>
</table>

### Table 2. CMFDA labeled cells – no fluorescence was detectable after 14 days in all three chondrocyte types.

<table>
<thead>
<tr>
<th>Day</th>
<th>Articular Cells</th>
<th>Auricular Cells</th>
<th>Costal Cells</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>85.8%</td>
<td>83.4%</td>
<td>89.1%</td>
</tr>
<tr>
<td>14</td>
<td>43.2%</td>
<td>38.2%</td>
<td>37.2%</td>
</tr>
<tr>
<td>21</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>
Table 3. Number of cells and daughter cell generations in flasks at time points of passage. The CMFDA labeled articular and costal chondrocytes were not confluent when no labeling could be observed any longer. Controls represent unlabeled costal chondrocytes. The replication of the cells is not inhibited by the dye.

<table>
<thead>
<tr>
<th>Labeling method</th>
<th>PKH 26®</th>
<th>Chondrocyte type</th>
<th>Articular</th>
<th>Auricular</th>
<th>Costal</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passage #</td>
<td>Cell #</td>
<td>Generations</td>
<td>Cell #</td>
<td>Generations</td>
<td>Cell #</td>
<td>Generations</td>
</tr>
<tr>
<td>1</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
</tr>
<tr>
<td>2</td>
<td>1x10^6</td>
<td>2</td>
<td>1x10^6</td>
<td>2</td>
<td>1.5x10^6</td>
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<td>2</td>
<td>1x10^6</td>
<td>2</td>
<td>1x10^6</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Labeling method</th>
<th>CMFDA</th>
<th>Chondrocyte type</th>
<th>Articular</th>
<th>Auricular</th>
<th>Costal</th>
<th>Controls</th>
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<tr>
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<td>Cell #</td>
<td>Generations</td>
<td>Cell #</td>
<td>Generations</td>
<td>Cell #</td>
<td>Generations</td>
</tr>
<tr>
<td>1</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
<td>0.4x10^6</td>
</tr>
<tr>
<td>2</td>
<td>--</td>
<td>--</td>
<td>1.5x10^6</td>
<td>3</td>
<td>--</td>
<td>2x10^6</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Figure 1. Seeded articular chondrocytes in flasks chondrocytes, labeled by PKH 26® and CMFDA show a loss of fluorescence over time. Time in days (time x d) Controls show no staining. Fluorescence microscope, 40x magnification.

Table 3. Number of cells and daughter cell generations in flasks at time points of passage. The CMFDA labeled articular and costal chondrocytes were not confluent when no labeling could be observed any longer. Controls represent unlabeled costal chondrocytes. The replication of the cells is not inhibited by the dye.

3.2. Transplanted Chondrocytes in Vivo

The constructs of PKH 26® and CMFDA stained chondrocytes in fibrin glue were harvested after 3 and 12 weeks. The samples were examined morphologically and histologically under a fluorescent microscope. The samples showed cartilage-like gross macroscopical appearance and had the consistency of cartilage. Under microscopic examination the fluorescence of PKH 26® labeled chondrocytes was bright and clearly distinguishable from the background (Figures 2, 3, 4, 5), even after 12 weeks in vivo after implantation. All three types of chondrocytes revealed the same pattern of fluorescence. Neo cartilage was formed and the daughter cells were tracked due to fluorescing.

The constructs containing CMFDA labeled chondrocytes of all three sources did not reveal fluorescence after
3 and 12 weeks. None of the controls showed fluorescence (Figure 5).

4. DISCUSSION

In vitro and in vivo cell tracking is important to distinguish the origin of newly formed tissue. Data have been collected on different labeling techniques, such as BrdU – bromodesoxyuridine [7, 15], labeling of cells with [3H] thymidine prior to transplantation [2, 14, 15], or transfection with the LacZ gene or green fluorescent for the expression of beta – galactosidase [6, 12, 22]. These techniques have some method specific disadvantages. BrdU is a relatively expensive agent that depends on the synthesis phase of the cell cycle for integration and an additional secondary antibody is needed to detect labeled cells. In addition there is little information about the longevity of the labeling in cells. [3H] thymidine is also cell cycle dependant and labeled cells have to be incubated for 2 months in order to detect the labeling [2, 14, 15]. The use of viral vectors to transport LacZ as a marker gene into cells requires specific timing for use and might cause unknown effects onto cells, such as immune reactions. Also, extra safety methods are required when using Adenovirus as a vector and the cell staining might last through only a few generations of daughter cells [6].

Other methods offered on the market are lipofection, which comes as a complete kit, but the transfection rate is low and the kits are rather expensive [16].

Thus, our goal was to identify labeling techniques that are stable with time, easy to use, and highly effective in cell labeling and cell staining for several generations culturing. They should also not be dependant on cell cycles for labeling chondrocytes for a long-term study.

In this long-term in vitro and in vivo study we evaluated the durability and staining differences of two newer labeling methods in chondrocytes: PKH 26® and CMFDA. We labeled three different sources of cells and showed that there was no qualitative difference in labeling between articular, auricular and costal chondrocytes, using either PKH 26® or CMFDA. However, PKH 26® proved to be more durable in labeling than CMFDA over 63 days. This result was also found in our previous in vivo experiment, tracking transplanted chondrocytes in a meniscus [27]. All three types of chondrocytes were labeled under the same conditions at the same time. We did not observe any difference in the fluorescence of different chondrocytes at the beginning of the study, although a higher cell number of fluorescent articular chondrocytes was observed at day 63 in comparison to auricular or costal cells. The fluorescence of PKH 26® labeled chondrocytes was stable in vitro until day 63, when only single cells showed fluorescence and the third passage was achieved. The longest lasting period for PKH 26® labeled cells in vivo was reported to be 4 months for neural cells post transplantation into the caudate putamen [9]. Other groups tracked labeled lymphocytes or peripheral blood mononuclear cells for shorter periods of time [3, 8, 10, 19], as well as neuronal precursors and neuronal cells [9, 23], endothelial cells [18], lymphocytes [20], L9292 cells [21] and hematopoetic stem cells.

In contrast to PKH 26® CMFDA labeled chondrocytes revealed fluorescence only until day 14, consistent with findings for myocytes, keratinocytes and osteocytes [3]. After that, no further fluorescence could be detected.
Yang et al. labeled osteoprogenitor cells with CMFDA and fluorescence was visible until 4-6 weeks of in vitro culture [27]. This might indicate that lasting of fluorescence depends on the cell source labeled. The manufacturer states bright labeling will last for at least 72 hours (Molecular Probes, Product information 17-June-2002). We found similar results in our experiment.

Under high intensity illumination used for fluorescence microscopy irreversible destruction of the excited fluorophore often becomes the limiting factor for a long term follow up. This “photo bleaching” has been described by Song et al. [25] as photochemical reaction pathways; some involving reactions between adjacent dye molecules. Although some effective “antifade” reagents are available the majority of them are incompatible with living cells [11]. Photo bleaching might explain the quick fading of the CMFDA labeled chondrocytes. PKH 26® were more stable when exposed to high intensity fluorescent light.

Tracking of different cells in vivo is described to after PKH 26® i.v. infusion [1, 17, 19, 24]. Direct in vivo staining of lymphocytes with PKH 26® is possible by intramuscular injection [19]. For our in vivo experiments we chose to label the chondrocytes in vitro and then implant the cells suspended in hydrogel, according to the technique described by Yang et al. [27] for osteoprogenitor cells and Lee-MacAry et al. for splenocyte effector cells [13]. Fluorescence was observed in the neo cartilage after 3 and 12 weeks in vivo. Fluorescence of the cells was clear and distinguishable at both time points, confirming the passage of the fluorescence dye by cell division to daughter cells. Thereby durable tracking of transplanted cells is provided and origin of the newly formed tissue could be defined to be from the transplanted chondrocytes. All three types of chondrocytes revealed stable fluorescence within the newly formed tissue. We did not observe any qualitative differences in fluorescence between the three cell types.

So far, only few data exist on the use of CMFDA or PKH 26® for labeling chondrocytes in vitro or in vivo. Further studies are necessary to evaluate the effects of this long lasting dye on chondrocytes on the cellular level. This study demonstrated that PKH 26® is the preferable marker for chondrocytes in a long-term study when tracking daughter cells. It can reliably label up to 63 days or 3 passages of cells, and serve as an indicator for the source of neo-cartilage in tissue-engineered constructs in vivo.

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REFERENCES


Brain initiated interaction

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ABSTRACT

Brain-Computer Interfaces (BCI) are developed to help locked-in patients, who lose control of their bodies and are unable to perform simple tasks such as speech, locomotion, and can’t even effectively interact, with their environment. BCI shows promise in allowing these individuals to interact with a computer using EEG. A Brain Computer Interface is a communication system in which messages or commands that an individual sends to the external world do not pass through the brain’s normal output pathways of peripheral nerves and muscles. A system is created to allow individuals with motor disabilities to control the motion of the bed on which they are bedridden via BCI for drug delivery and other activities, with the help of eye motion and changes in the absolute power in alpha rhythms of an EEG signal of the patient.

Keywords: BCI, eye events, EEG, Lab VIEW

1. INTRODUCTION

BCI (Brain Computer Interface) research is a multidisciplinary field requiring the knowledge of neuroscience, physiology, psychology and engineering. For the development of BCI, we generally use the Electroencephalogram (EEG). EEG signal is composed of electrical rhythms and transient discharges. Features like wave shape, amplitude, frequency and power are detected which are typical for a particular act and can vary from person to person. Once these features are detected, they can be used to generate a control signal by using Translation algorithm and can be used to operate some devices. Brain-Computer Interface (BCI) shows a great potential to provide new channels for physically disabled people, especially locked in patients, to communicate and interact with the outside environment. EEG-based BCI is non-invasive, so it is more readily accepted. In this paper, we introduce the design of a BCI based on changes in EEG amplitude due to eye activity and in the absolute power of alpha rhythms after eye activity, followed by applications based on this core technology i.e. controlling the motion of the bed of severely paralyzed patients for drug and food delivery etc with the help of a stepper motor installed for controlling the motion of the bed in both directions i.e. up and down.

2. SYSTEM ARCHITECTURE

Figure 1 shows the complete experimental setup of a BCI system, designed to control environment (in this case motion of the bed).

The system is developed using virtual instrumentation technology and consists of basic two modules: hardware and software. The hardware set up of the system consists of

- EEG equipment (Head box and adaptor box)
- Desktop PC interfaced to EEG Hardware via USB port.
- USB based digital output signal interfacing board (National Instruments 6015) for motion control.
The software module was developed program for BCI using LABVIEW to perform the function of EEG acquisition analysis and display.

2.1. EEG Equipment

The device used for study is RMS 32 Brain View Plus. It can record 32 channels of EEG data from electrodes placed according to the international 10-20 system. The voltage generated by the brain cells and picked up by EEG is extremely small (between 10-20 microvolt) and amplification is needed of the order of ten thousand times for successful recording of the EEG signal. The odd numbered electrodes are placed on the left side of the head while even numbered electrodes are placed on the right side of the scalp. The view of the electrode positions as seen from the side and top is as shown in the following figure.

The standard parts of the EEG hardware include adaptor box, head box, connecting cable and PC. The Head Box is used for connecting electrodes from the scalp to the hardware unit. The signal generated is amplified and then sent to adaptor Box for signal conditioning. The digital signal generated then, passes to the PC where it is displayed on the screen on Super Spec software designed for display of EEG Signals.

2.2. Software Design of BCI

Software for the Brain computer interface is designed on the Lab VIEW platform which consists of software front panel for user interaction and block diagram programming code to control the overall functionality of the system. Figure 3 shows the functional elements of BCI system.

For easy understanding and debugging, the software code is divided into three sub modules namely:
- EEG signal acquisition and processing module
- Feature extraction module
- Device control module

EEG signal acquisition and processing module acquires online EEG signal from EEG machine channel (FP2-F4), (FP1-F3) the channel being more sensitive to eye events. Also the signal is acquired from (O2-CAR), CAR being the Common Averaged Reference, the channel most sensitive for variations in power of alpha rhythms. The raw sampled EEG data file created by EEG machine Super Spec software at the sampling rate of 256 Hz is then read continuously at the start of acquisition. The raw data is then processed using as series of filters. The signal is fed to a band pass filter implemented using low pass filter (4th order FIR filter with cut off 99Hz) and high pass filter (4th order FIR filter with cut off 0.1Hz) to limit the EEG signal bandwidth (0.1 to 99 Hz). A 50 Hz notch filter is used to remove power line interference. The processed EEG data is fed to feature extraction module which executes an amplitude and time-duration-based algorithm to detect the changes in the EEG signal due to eye events such as eye open and eye close. Once the event of eye open and eye close is detected then checks the absolute power in the signal of (O2-CAR) channel in the frequency range of 8 to 12 Hz of 512 samples with the help of FFT. If the event eye open is detected and then the power in the frequency range of 8 to 12 Hz is less than 0.5 V^2 the device control module is executed to send a high Boolean data type signal to a switch connected to digital output line P03 of USB based interfacing board through DAQ assistant that moves the stepper motor in anti clockwise direction for 33 steps. Similarly If the event eye close is detected and the power in the frequency range of 8 to 12 Hz is more than 2.5 V^2 of 512 samples the device control module is executed to send a low Boolean data type signal to a switch connected to digital output line P03 of USB based interfacing board through DAQ assistant that moves the stepper motor in clockwise direction for 33 steps. If the condition for particular event is not met the DAQ assistant is configured to send simultaneously the low Boolean data type signal at the particular output digital lines to make the bed remain in rest position. Once the stepper motor has taken 33 steps for the bed motion no further action is taken for a time period of 5 minutes what so ever may be the signal changes i.e. ACQ switch is made OFF. After 5 minutes Acquisition again starts and control action is taken accordingly.

The overall software program functionality is controlled by the customized design of soft front panel using controls and indicators, on PC screen, through which the user interacts with the BCI system. It consists of ‘ACQ switch’ to start and stop the acquisition of EEG signal data file, ‘EEG recorder’ a calibrated waveform chart to show graphical record of EEG signal at the time of acquisition of (FP2-F4 and O2-CAR) and one virtual bed
that can be moved up and down for 33 steps to depict the status of the particular eye event and power in the alpha rhythms.

3. RESULTS

Many factors determine the performance of a BCI system. These factors include the brain signals measured, the signal processing methods that extract signal features, the algorithms that translate these features into device commands, the output devices that execute these commands, the feedback provided to the user and the characteristics of the user. The parameters of the features extracted vary from individual to individual so it is important to develop the generalized BCI. The changes in the EEG due to eye motion are detected from the waveforms originating at FP2-F4, FP1-F3. The amount of change in the amplitude during eye open and eye close vary from subject to subject, location of electrodes on the forehead, physiological state of the patient and contact impedance of the electrodes on the scalp. The wave can be reconstructed and hence can be used for further control action in the development of BCI. The results are taken online. As soon as the BCI is switched on, the EEG pattern from the machine is recorded on the front panel and changes due to eye events are detected and displayed on the front panel. The changes in the EEG patterns are detected and intelligent control action is taken we found that the production of changes due to eye events is not the same for all the cases. For some person the amplitude is different, for some latency is different. To remove this problem we used a sensitivity factor. The function of the sensitivity factor is to vary the threshold values for the eye events. Normally it is observed that if we keep a factor of 250 on positive side and –125 on negative sides the detection is almost clear. In some cases we have to increase sensitivity factor. A factor of 100 is provided which seems to work best. If we increase the sensitivity the factor on positive side now goes to 350 and on negative side it goes to −225. Similarly we can further increase or decrease the sensitivity factor depending on the patient’s context.

4. CONCLUSION AND FUTURE SCOPE

The key is to take BCI technology beyond the demonstration stage to the real world applications, so that the quality of life for paralyzed patients is improved. We detected the changes in the EEG patterns due to eye events. We have used eye events and power in the alpha rhythms for control of bed motion to facilitate the drug and food delivery to the patients. The possibility of expanding the BCI into latest technology will enhance the adoption of this technology and develop into feasible solutions with further advances. It can be further used to design a virtual keyboard which can enable the locked in patients to interact with PC.

REFERENCES

Left ventricular systolic function assessment in patients with dilated heart failure using cardiovascular magnetic resonance

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ABSTRACT

Cardiovascular magnetic resonance (CMR) has become a reference standard for the measurement of cardiac volumes, function, and mass. This study aims to reconstruct three-dimensional modeling of the left ventricle (LV) in patients with heart failure (HF) using CMR tools and thereby derive the LV functional indices. CMR images were acquired in 41 subjects (6 females) with heart failure (HF) and 12 normal controls (4 females). Five comparisons were made (i) normal and dilated heart failure subjects, (ii) male and female normal heart, (iii) male and female dilated heart, (iv) male normal and dilated heart failure and (v) female normal and dilated heart failure. In HF, a significant higher values of EDV (320 ± 79 vs. 126 ± 22 ml, P<0.0001), ESV (255 ± 68 vs. 54 ± 12 ml, P<0.00001) and lower values of EF (20 ± 7 vs. 58 ± 5 %) were found compared that of normal control. There were significant difference on LV EDV and ESV between sex in both normal and HF subjects.

Keywords: Dilated heart failure, magnetic resonance imaging, left ventricle, systolic function

1. INTRODUCTION

The heart is divided into right and left sides by a septum, which is a partition consisting of myocardium covered in endocardium. Each side is further divided by the atrioventricular valve (AV) into upper chamber, the atrium, and lower chamber, the ventricle. The AV valves are formed by double folds of endocardium strengthened by fibrous tissues. The right atrioventricular valve (tricuspid valves) has three flaps or cusps and the left atrioventricular valve (mitral valve) has two.

The heart, with its action as a pump in mechanical analogy, produces series of events within a period of time known as the cardiac cycle. During each cardiac cycle, the heart contracts and then relaxes creating the systolic and diastolic pressures. Contraction of the heart expels a percentage of oxygenated blood known as the cardiac output, given as product of heart rate and stroke volume. The period of contraction is called systole and that of relaxation, diastole.

The valves between the atria and ventricles open and close passively according to the pressure in the heart chambers. They open when the pressure in the atria is greater than that in the ventricles. During ventricular systole (i.e., contraction), the pressure in the ventricles rises greater than in the atria, thus shuts the valves to prevent backward flow of blood. The valves are prevented from opening upwards into the atria by tendinous cords, which extend from the interior surface of the cusps to little projections of myocardium covered with endothelium, called the papillary muscles.

The muscle layer of the walls of the atria is very thin compared to the ventricles. This is consistent with the amount of work it does. The atria, assisted by gravity, only propel blood through the AV valves into the ventricles. The ventricles on the other hand, actively pump the blood to the lungs and to the distal parts of the body. In the case of the left ventricle, oxygenated blood from the left atria enters the left AV valve into the left ventricle, and from there the blood is pumped via the aorta, then the peripheral arteries and to different organs. Therefore the muscle layer is thickest in the wall of the left ventricle.

When the pressure developed in the left ventricle by the contraction of myocardium is less than the pressure in the aorta, the ventricle cannot pump out the normal amount of blood resulting in left ventricular failure. This phenomenon can be caused by excessively high systemic (aortic) blood pressure, incompetence of the mitral and/or the aortic valve, aortic valve stenosis and myocardial weakness. Failure of the left ventricle leads to dilatation of the atrium and an increase in pulmonary blood pressure. This is followed by a rise at the blood pressure in the right side of the heart and eventually systemic venous congestion.

1.1. Comparison of Imaging Techniques on Cardiovascular Disease Diagnosis
Over the past decades, the ability of technology to diagnose heart disease has improved dramatically, largely due to the evolution of new techniques such as echocardiography, nuclear cardiology, cardiac computed tomography and cardiovascular magnetic resonance [1]. When patients complain of signs and symptoms such as chest pain, shortness of breath, and an abnormal pulse, further diagnosis can then be administered. Further examinations and quantification of the left ventricular (LV) ejection fraction (LVEF), LV end-diastolic volume (LVEDV), and LV end-systolic volume (LVESV). These are important prognostic parameters in patients with chronic coronary artery disease (CAD) and LV dysfunction [2]. Accurate assessment of LVEF and LV volumes in these patients is important, and several imaging techniques are available for this purpose such as the echocardiography, cardiovascular magnetic resonance, Nuclear Cardiology and computed tomography. The advantage and disadvantage of these methods are briefly discussed as follow.

1.2. Echocardiography Methodology

Echocardiography is one the noninvasive techniques used in the diagnosis of heart disease. Echocardiograms are obtained by reflecting high frequency sound waves off various structures of the heart, then translating the reflected waves into one- and two-dimensional images. Echocardiography can produce detailed three-dimensional images of the heart’s anatomy [7, 10]. These images can used to estimate heart size, functionality, and wall thickness of the muscle. With the combination of Doppler technique, echocardiography can be used to estimate blood flow through the heart chambers and the pressure gradients across valves to determine the degree of narrowing, regulation, or ventricular calcification. When combined with stress test, echocardiography is able to evaluate wall motion of the ventricles and other physical characteristics of the heart under stress.

Echocardiography can also be used to detect tumours or clots within the heart and other congenital abnormalities. It is completely non contact, therefore eliminates any pain or risk during testing. The concern of using this technique is that, echocardiography cannot measure ejection fraction as precise as other imaging techniques especially for patients with broad chests or obese [8, 11].

1.3. Nuclear Cardiology Methodology

The use of radioactive substances to examine the function of the heart was first introduced as early as 1927 [1]. The analysis was by injecting a small amount of a short-lived radioisotope into the bloodstream and then track its progress and specific uptake in the circulatory system using a radiation-detecting device. During nuclear cardiology procedures, a scintillation gamma camera is used to detect the radiation (gamma rays) emitted by the isotope, the data is collected and processed by computer. Information is then quantified to display as a picture of the heart.

Nuclear cardiology provides accurate measurement of heart function especially the ejection fraction [2, 4, 5]. However, quality of data can be affected for patients with irregular heart rhythm. This technique is not suitable for pregnant women and nursing mother.

1.4. Cardiac Computed Tomography

Cardiac Computed Tomography is also known as single photon emission computed tomography (SPECT). SPECT can be used to obtain three-dimensional thallium images of the heart. It is superior in detecting individual lesions in the coronary arteries and to identify the location of damaged and ischemic heart muscle. SPECT has been used to assess the effects of treatment for ischemic heart disease [9]. Although Cardiac Computed tomography is also used to diagnose stroke, its use in cardiovascular disease is mainly confined to diseases of the aorta. The cost of using SPECT is high compared to other standard nuclear imaging techniques.

1.5. Cardiovascular Magnetic Resonance

Cardiovascular magnetic resonance (CMR) images are acquired with patient in supine position by using the 1.5-T MRI system with a flexible body array coil for signal reception. Spin-echo scout images are obtained in the coronal and sagittal imaging planes. It enables CMR to provide accurate, reproducible assessment of cardiac function through acquisition of tomography images of high spatial and temporal resolution. CMR is free of ionizing radiation.

LV functional indices will be analysed in this study. They are (i) end-diastolic Volume (EDV), (ii) end-systolic volume (ESV), (iii) ejection fraction (EF) and (iv) stroke volume (SV). They are defined as: 

- End-diastolic volume (EDV) – EDV is the volume of blood in the ventricle at the end of filling (i.e., end-diastole). An increase in EDV increases the amount of blood ejected from the ventricle during systole (i.e., stroke volume).
- End-systolic volume (ESV) – ESV is the volume of blood in the ventricle at the end of the cardiac ejection period and immediately preceding the beginning of ventricular relaxation. Measurement of the adequacy of cardiac emptying relates to systolic function. End-systolic volume will be seen at the end of the T wave.

Ejection fraction (EF) – EF is the fraction of blood ejected by the ventricle relative to its end-diastolic volume (i.e., \( EF = SV/EDV \times 100\% \)).

Ejection fraction is commonly measured using echo-cardiography. This non-invasive technique provides good estimates of EDV, ESV, and SV. Normally, EF is >60%. EF is often used as a clinical index to evaluate the inotropic status of the heart. A high EF could indicate the presence of certain heart conditions, such as hypertrophy cardiomyopathy [1]. A low EF could be a sign that the heart is weakened [1]. One should be aware that, it is important to note that there are circumstances in which EF can be normal, yet the ventricle is in failure. One example is diastolic dysfunction caused by hypertrophy in
Figure 1. Cine-frame images of a 4-chamber (above panel), a short-axis (middle panel) and 3-dimensional reconstruction (below panel) of left ventricle at end-diastolic (left panel) and end-systolic (right panel) phases. 3-dimensional reconstruction is for calculation of ventricular volumes and function.

Table 1. LV functional Indices between the normal and HF Subjects.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Heart failure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDV (ml)</td>
<td>ESV (ml)</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>126 ± 22</td>
<td>54 ± 13</td>
</tr>
<tr>
<td>Min</td>
<td>81</td>
<td>30</td>
</tr>
<tr>
<td>Max</td>
<td>156</td>
<td>78</td>
</tr>
</tbody>
</table>
which filling is impaired because of low ventricular compliance and stroke volume is therefore reduced. In the case of dilated HF, the value of EF becomes very small as SV decreases and EDV increases. In severe HF, EF may be 20% or less.

Stroke volume (SV) – SV is the amount of blood pumped by the LV in one contraction. Normally, only about two-thirds of the blood in the ventricle is pumped out with each beat. What blood is actually pumped from the left ventricle is the stroke volume and it, together with the heart rate, determines the cardiac output.

2. METHOD AND DESIGN

53 subjects were included in the study from two groups: 41 patients with dilated HF and 12 normal volunteers. CMRtools was used to reconstruct the 3-dimensional (3D) model of the LV and to derive LV functional indices (i.e., EDV, ESV, SV and EF). One sample 3D model of the LV was shown in Figure 1. 2-sample t-test was performed to assess any significant differences on LV functional indices between normal and dilated HF subjects. A commercially available statistical software package was used for data analysis (Minitab 14).

Table 2. The P values of comparisons.

<table>
<thead>
<tr>
<th>P value</th>
<th>EDV (ml)</th>
<th>ESV (ml)</th>
<th>SV (ml)</th>
<th>EF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs. HF</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.224</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal vs. HF in male</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.062</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal vs. HF in female</td>
<td>0.001</td>
<td>0.001</td>
<td>0.516</td>
<td>0.001</td>
</tr>
<tr>
<td>Male vs. Female in normal</td>
<td>0.012</td>
<td>0.066</td>
<td>0.002</td>
<td>0.965</td>
</tr>
<tr>
<td>Male vs. Female in HF</td>
<td>0.004</td>
<td>0.010</td>
<td>0.224</td>
<td>0.635</td>
</tr>
</tbody>
</table>

LV 3-dimensional modeling was reconstructed and the LV functional indices were derived. It was seen that the mean value of EDV is 126 ml in normal subjects compared to 320 ml in the dilated HF subjects. For ESV, the values increased from 54 ml in normal subjects to 255 ml in dilated HF subjects.

Various studies have been done to compare the functional indices between MRI and various other medical imaging available when diagnosing the normal and heart failure [2, 3, 4, 6, 8, 12]. Riemer et al [2] reported their MRI study conducted on 38 patients with chronic coronary diseases that the values of EDV range from 61 ml to 267 ml, ESV from 31 ml to 202 ml and EF from 14% to 59%. Compared to PET where the EDV value is 41 – 242 ml, ESV 24 – 198 ml and EF 13 – 55 %. Only ESV showed no significant difference between MRI and PET. Tuncay Hazirolan et al [8] compared their MRI with echocardiography on 20 patients with 15 reported history of myocardial infarction and showed that there is no significant difference between ESV and EF for dilated HF. The EDV and SV values from MRI were higher compared to the measurement from echocardiography. Lissa Sugeng et al [11] compared CT with MRI with 31 subjects (14 female) and found 9 of which were normal and their result showed that the measurement with CT resulted in significantly overestimation of both EDV and ESV. Based on findings from these studies, we found that CMR has no significant difference when compared to PET and echocardiography on LV volumes, but not for CT. EDV, ESV and EF values obtained from our study fall within the range previously reported by other authors [2, 11].

5. CONCLUSION

This study showed a significant difference on EDV, ESV and EF between normal and HF subjects.

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Enhanced apoptosis and electrostatic acetylcholinesterase activity of abnormally hydrophobic environment in Alzheimer’s plaques

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ABSTRACT

Alzheimer’s disease (AD) is considered a slow neuronal dysfunction process through hypoxia, ischemia and leads to apoptosis mediated senile plaques and neurofibrillary tangles (NFTs). Due to non-invasive approach of plaque characterization, computational techniques based on Brownian dynamics simulation are unique to speculate the electrostatic and kinetic properties of Acetylcholinesterase (AChE). Typically the MRI spectroscopy high choline peak and enzyme specific to Alzheimer’s Disease (specificity constant \( k_{cat}/K_m \) of AChE) appeared associated with apoptosis and hypoxia. A simple display between synergy of cytokines, apoptosis, elevated AChE and choline is postulated as initial events. The events may be distributed heterogeneously within the senile plaques and neurofibrillary tangles (NFTs) of Alzheimer’s Disease (AD). The role of decreased brain AChE and synergy was associated with specific Magnetic Resonance Spectroscopic (MRS) pattern profiles in AD. These findings suggest that the altered AChE and early apoptosis events in AD may be associated with specific MR spectral peak patterns. This study opens the possibility of reduced AChE levels causing high choline and reduced N-acetyl acetate (NAA) neurotransmitter by MRS after initial apoptosis and/or inflammation to make amyloid plaques in the cerebral tissue of Alzheimer’s disease (AD) patients. These results can be useful in clinical trials on AD lesions.

Keywords: Alzheimer’s Disease, Acetylcholinesterase, Electrostatics, Dielectric effect, Ionic effect, Brownian dynamics, Apoptosis

1. INTRODUCTION

1.1. Alzheimer’s Disease

Alzheimer’s disease (AD) has manifestations of senile plaques and neurofibrillary tangles (NFTs) in the cerebral cortex involving hippocampus of Alzheimer’s brains. Many studies have shown that the levels of the reduced ACh neurotransmitter in AD brain had been curiosity in recent past. In this direction, promising success is claimed in drug therapeutic trials based on AChE inhibitors. In AD presumably five neurological cell groups are commonly seen around the cortex rich with AD lesions associated with the low AChE enzyme. Other important problem of AD therapeutics was solved by use of AChE inhibitor higher concentrations such as, tacrine, physostigmine, and BW284C51 to inhibit AChE within AD lesions. Simultaneously, elevated levels of the ACh substrate also inhibit AChE activity. Serotonin, 5-hydroxytryptophan, carboxypeptidase inhibitor, and bacitracin, had been good choice to effectively inhibit cholinesterase activity within plaques and tangles, but fail to alter the AChE activity in normal tissue at standard physiological conditions. The initial stages of amyloid plaque formation are not known if they are the result of metabolic defect leading to pathology. The mechanisms were reviewed by Lu et al. 2003 [12]. The process of inflammation in AD was described by Bamberger et al. 2002 [5]. There is continuous hunt of biomarkers useful in AD reported by Ankarcrona et al. 2002 [3]. However, the sequence of these events remains unknown. There are several reports showing that neurons die partly by apoptosis in the AD brain. Drugs blocking apoptosis could therefore be potentially useful for early prevention of neuronal cell death. Biomarkers for apoptosis should be important tools in the evaluation of drug effects and in the diagnostics of AD. Future strategies are more likely to modify the course of the disease. The mos widely accepted hypothesis on the etiopathogenesis of AD proposes that aggregates of beta amyloid (Abeta) form in the brain. Under normal conditions, the predominant amyloid peptide secreted is Abeta(1-40) with about 10-15% being the longer 1-42 form. In AD, there appears to be an increase in the longer more toxic form which is proposed to trigger tau hyperphosphorylation and neural degeneration. Neurotoxicity is thought to be due to altered calcium regulation, mitochondrial damage and/or immune stimulation. One strategy for treating AD is the prevention of Abeta release or the blockade of it neurotoxic activity re-
ported by Lu 2003 [12].

Present paper explains the electrostatics of lowered AChE catalytic activity in AD brain tissue over normal tissue with possibility of the protein-rich deposits associated with the onset of AD. The paper further, illustrates the possibility of reduced AChE associated with high choline and reduced NAA peaks by MRS and initial events triggered by cytokines, apoptosis and inflammation to synthesize amyloid protein as NFT plaques. However, the high concentrations of protein-rich deposits in plaques and NFTs such as βAP, heparan and dermatan sulfate proteoglycans, serum amyloid P component, complement factors, and protein kinase C, had been active research over the increased the hydrophobicity of AChE in AD lesions and abnormally reduced dielectric constant reported by Giambriglia et al. 1997 [9]. The AChE catalysis has been explained as electrostatic steering mechanism where altered dielectric conditions seen by βAP deposition. Possibly, dielectric constant shift in AD tissue also allows Coulombic interactions to permeate longer distances resulting with enhanced enzymatic activity and simultaneously decreased ACh levels. MR spectral pattern of enhanced choline also supports association with decreased ACh levels in AD.

1.2. Acetylcholinesterase

The acetylcholinesterase enzyme (AChE) has 537 amino acid long polypeptide in the postsynaptic neural membranes of central nervous system and neuromuscular junctions by a glycosylphosphatidylinositol linkage. AChE catalyzes the hydrolysis of the acetylcholine (ACh) substrate neurotransmitter at cholinergic synapses. AChE hydrolysis results in the termination of impulse transmission.

The determination of the three-dimensional structure of AChE dimer enzyme comprises 12-stranded mixed β-sheet surrounded by 14 α-helices. These subunits assemble through disulfide linkage and hydrophobic interactions. The enzyme structure shows structural characteristic of AChE as a deep (~20Å), narrow active site making enzyme’s catalytic site Ser$^{200}$, His$^{440}$, and Glu$^{267}$ at its base. The walls of this entity are lined with 14 highly conserved aromatic amino acids of active site. Positively charged ACh substrate toward the active site caused low-affinity cation-π interactions. Further, amino acid charge distribution over AChE creates an electric field around the enzyme contributing to its enzymatic activity (electrostatic steering mechanism) involving its substrate, ACh [12]. Authors determined that the negative field drives the positively-charged ACh substrate molecule toward the entrance of its active site moiety and increases the catalytic rate of AChE.

1.3. Cytokines, Inflammation, Apoptosis, and Serum AChE Relationship in AD

Inflammatory processes play a role in disease progression and pathology of AD, which involves the deposition of amyloid in the brain and the extensive loss of neurons. Amyloid plaque deposition is accompanied by the association of microglia with the senile plaque, and this interaction stimulates these cells to undergo phenotypic activation and the subsequent expression of proinflammatory cytokines and neurotoxic products [5]. Inflammation has been reported in numerous neurodegenerative disorders such as Parkinson’s disease, stroke and Alzheimer's disease (AD). In AD, the inflammatory response is mainly located to the vicinity of amyloid plaques. Cytokines, such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), Tumor Necrosis Factor alpha (TNF-α) and Transforming Growth Factor beta (TGF-β) have been clearly involved in this inflammatory process. Although their expression is induced by the presence of amyloid-beta peptide, these cytokines are also able to promote the accumulation of amyloid beta peptide. Altogether, IL-1, IL-6, TNF-α and TGF-β should be considered as key players of a vicious circle leading to the progression of the disease reported by Cacquevel et al., 2004 [6]. Inflammatory stimuli also induce nitric oxide production, resulting in oxygen deficiency (hypoxia) and stimulating adenylate cyclase activity. Under these conditions, the rate of apoptosis increases. Neuron dysfunction is partly due to apoptosis in the AD brain (Figure 1).

2. RESULTS AND DISCUSSION

2.1. Ionic Strength

Computed rate constants of Torpedo californica AChE as model enzyme at various ionic strengths are given in Figure 2. These values are compared with experimental bi-molecular association constants ($k_{cat}/K_m$) and enzymatic specificity constants ($k_{cat}/K_m$) of a related Electrophorus electricus AChE enzyme as reported by Nolte et al., 1980 [14]. Since the association constant considers the binding event of the reaction and the specificity constant describes both binding and subsequent catalytic turnover, $k_{f}$ is the theoretical maximum value for the calculated diffusion-controlled rate constant, while $k_{cat}/K_m$ sets the lower limit on these second-order reactions. As seen in Figure 2, the calculated rate constants found in this work lie between these two extremes throughout the range of ionic strengths tested. This provides encouraging support for the ionic screening approximations used in this work. Furthermore, the decrease in the rate of AChE catalysis with increasing ionic strength provides strong evidence that an electrostatic steering mechanism plays a role in AChE kinetics. The similarity in the negative slope observed for both association and specificity constants indicates that the ligand binding step of the reaction is dependent upon solvent salt concentration.

2.2. Substrate Radius

To demonstrate the limited accessibility of this enzyme’s active site structure, simulations were performed using various substrate radii (Figure 2). It is reasonable to predict increased rate constant values with a reduced substrate radius since the probability of a smaller substrate penetrating the active site gorge and reacting with AChE is higher. The results shown in Figure 3 show this expected
Figure 1. A postulated schematic sketch of the relation among inflammatory cytokines, adenylate cyclase stimulation, AChE and cytotoxicity events in AD. (Solid lines represent stimulation effect and dotted lines represent inhibition effect of different effectors. Abbreviation: IKK; IκB kinase kinase, NF-κB; nuclear factor B, NF-κB-IκB; nuclear factor B-inhibitor B complex, NO; nitric oxide, iNOS; inducible nitric oxide synthase, SOD; superoxide dismutase, PKC; protein kinase C, GTP; guanosine triphosphate, GITP; guanylyl imidotriphosphate, ACh; acetylcholine, AChE; acetylcholinesterase enzyme. Possible sequence: Low OxygenÆ1. Low ATP (oxidative Phosphorylation); 2. Low Pyruvate (low N-Aceto-Acetate by glycolysis)ÆHigh Choline (Reduced AChE). Cytokines trigger adenylate cyclase to inflammation and amyloid protein synthesis ?? (mechanism is unknown).)

Table 1. Effect of Dielectric on Activity of wt AChE. 8000 trajectories were simulated at 300 K, an ionic strength of 5 mM, a solvent density of 996.5 kg/m³, and a pH of 7.0.

<table>
<thead>
<tr>
<th>Dielectric constant</th>
<th>Rate Constant M⁻¹s⁻¹</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>78</td>
<td>1.3 x 10⁹</td>
<td>± 0.267 x 10⁹</td>
</tr>
<tr>
<td>60</td>
<td>4.7 x 10⁹</td>
<td>± 0.615 x 10⁹</td>
</tr>
</tbody>
</table>

Comparing the electric field contours around the perimeter of AChE at dielectrics of 78 and 60 (Figure 2). These contour plots illustrate an electrostatic gradient emerging from the gorge entrance and extending along the enzyme’s surface. This effectively enlarges the active site target area, resulting in increased enzyme-substrate association. These electric field calculations along the protein’s surface are consistent with the results of Antosiewicz et al. 1995b [3], who have suggested that electrostatic steering is limited to operation near the surface of the enzyme. The contour plots found in this work also offer a plausible explanation for the catalytic rate constant enhancement at lower dielectrics and further suggest that electrostatic attraction is an important component of the AChE mechanism and ultimately its physiological role in the human nervous system.

The 3D MP-RAGE at TR/TI/TE=10/250/4 ms, flip an-
ble 15, 1.0 x 1.0 mm² resolution, and 1.4 mm thick partitions in our previous report showed NAA/Cr, NAA/Cho were significantly reduced (p < 0.02 and p value < 0.03 respectively) in AD compared with elderly controls due to reductions of NAA after NAA correction in AD. Furthermore, the difference of hippocampal NAA between the groups without atrophy correction (which reflects both NAA and volume changes) was about 40% larger than with correction of atrophy as shown in Figure 3. The major elevated peak was choline at 3.00 ppm. These finding suggest a possibility of reduced glycolysis leading to low N-acetyl acetate formation and choline accumulation indirectly reducing TCA cycle to generate enough ATP in localized tissue. Low ATP and oxygen are well understood to lead inflammation and amyloid plaque formation.

3. CONCLUSIONS

Abnormal Magnetic Resonance spectral NAA, choline peak patterns are associated with low AChE enzyme activity in AD with possible enhanced ACh breakdown and surrounding electrostatic field of this enzyme. However, the association of cytokines, apoptosis to lead hypoxia and inflammation in amyloid plaque in AD formation is unclear.

REFERENCES


Temperature and Magnetic Resonance Characteristics of Zinc, Manganese, Gadolinium, Gold, Iron Magnetic Nanoparticles and Cytokine Synergy in Hyperthermia

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ABSTRACT

The temperature sensitive magnetic resonance dependence for assessing localized heating effect of Manganese (Mn), Zinc (Zn), Gadolinium(Gd), Gold(Au) and Iron(Fe) magnetic nanoparticles was compared. These particles showed heating effect when subjected to alternating filed. The relationship between temperature and magnetic nanoparticle moment is specific in imaging. The art of imaging temperature in a tumor at various locations is emerging as the selective approach of hyperthermia to monitor temperature and treat the tumor. Two unsolved issues are related with tumor temperature rise in the presence of magnetized nanoparticles. First, the relationship of tumor energy changes as a result of cytokine synergy after tumor magnetization. The second issue is linear attenuation after magnetic field exposure with tissue temperature increase due to inflammation and lysosomal enzyme action in tumor. In present paper, a new approach of heating tumor is analyzed without spot heating by polymer coated particles at controlled Curie temperature of less than 44°C. The study reports a comparison of Mn, Zn, Gd, Fe, Au nanoparticles designed for imaging purpose using chemical co-precipitation technique. The possibility of nanoparticle stimulated temperature treatment (hypothermia effect) is hypothesized to recover the tumor metabolic integrity and inflammatory status of tumor cells presumably associated with the depleted intracellular energy (low ATP) and the inflammation (elevated cytokines, interleukins) and lysosomal enzymes initially. The multimodal imaging techniques were compared using nanoparticles for their sensitivity. The art of the nanoparticle induced hyperthermia does have a great impact on public health as alternative therapeutic oncology.

Keywords: hyperthermia, nanoparticles, temperature imaging, cytokine

1. INTRODUCTION

Heating tumors by nanoparticles and resistance in hypoxic tumor cells to a high temperature is emerging as an effective tool in therapeutic oncology [1]. Heating of organs and tissues in cancer treatment was first reported [2]. The introduction of nanoparticles enhanced the diagnosis and localization of specific tumor characteristics by multimodal imaging techniques including optical, magnetic resonance, positron emission tomography, computed tomography and X-ray techniques. With rapid development, feasible clinical therapeutic applicators and hyperthermia equipment were designed. In this direction, colloidal gold-thiol preparations were first reported as effective staining agents to label proteins in both diagnostics such as imaging, blotting, flow cytometry, hybridization assays and gold-thiol hyperthermia agents. Other potential hyperthermic particles are silver, iron, zinc and lanthanum nanoparticles [3-10].

At sites of tumor, hyperthermia is a state of therapeutic temperature induced recovery of metabolic integrity and enough defense by cytokines and lysosomes to compensate the damage perhaps increase in oxygenated blood circulation across tumors due to stimulus of heat. In normal tumor, the cells experience loss of metabolic integrity, hypoxia, inflammation, stimulated lysosomal enzymes due to phagocytosis. As a result tumor cells get overactive and overgrown. The hyperthermia can be produced by the interaction of tumor cells with energy such as alpha-, beta- or gamma radiation (Therapeutic Nuclear Medicine), X-Ray (radiotherapy), ultrasound (Therapeutic Ultrasonography) and magnetization (magnetotherapy). However, these interactions are not risk free. Alter
native common practice of radiation therapy puts hazard of oxygen radical formation and tumor’s cell DNA strand breakage. However, oxygen-starved hypoxic tumor cells are three-times more resistant to ionizing radiation than normal cells [8]. As a result, hypoxic cells show failure in achieving local tumor control through ionizing radiation hyperthermia treatments as shown in Figure 1 and reported elsewhere [9-10]. Good catch is here to enhance the magnitude of heating effect in tumor by either of the energy type using nanoparticles. This alternative approach can be useful in imaging such as “magnetic hyperthermia for MRI” or “gold hyperthermia for X-Ray” or “Acoustic hyperthermia for ultrasound” by introducing these particles uniformly distributed in tumors.

In recent years, these particles have been synthesized to magnify the tissue heating effect. However, hyperthermia may be hazard of spot overheating that causes necrosis. Magnetic hyperthermia is the method of heating body tissue using magnetic materials [11, 15, 16]. The overheating may be avoided by controlling the particles’ dissipating heating effect at their Curie temperature. Presently, colloidal gold particles are used in monitoring metabolic changes as are bound with antibodies, lipids, DNA fragments during cellular uptake and endocytosis. In human body, nanomagnetic particles with Curie temperature of 42-43ºC are most suitable as hyperthermia heat source at the tumor site exposed to an alternating field [11].

In our lab, a simple resonant circuit was developed for nanoparticles heating effect after applied alternating fields to generate hyperthermia as shown in Figure 2. The particle behave is specific to Curie temperature. At Curie temperature, particles generate consistent heat at the applied alternating fields to maintain tumor heating [14-15]. Mn-Zn-Gd Ferrite nanoparticles have advantage as their properties can be desirable by varying the proportions of the constituent Mn or Zn or Gd elements [17-19].

In this feasibility paper, our focus is on the use of different techniques that were used in our lab. Such techniques are: 1. synthesis, targeting the tumor and the relaxation characteristics of nanoparticles in the tissue; 2. Mechanistic approach of cytokines and lysosomal enzymes in tumors and imaging the nanoparticle concentrations in the tumors; 3. Comparison of newer nanoparticles in hyperthermia and emerging biomedical applications. This study further demonstrates three main interactions contributing the measurable signal: 1. Tumor tissue relaxation behavior and temperature dependence; 2. Nanoparticles relaxation behavior and temperature dependence; 3. Intracellular tumor cell biochemical- NMR relationship. The paper also shows a comparison of manganese, zinc, gadolinium, ferrite, gold and lanthanum nanomagnetic particles and their magnetic moment dependence on temperature to highlight the significance of Curie temperature of 42-43ºC in hyperthermia. The emerging newer concepts are revisited if cytokine synergy and energy metabolic integrity of tumor cells participate in temperature rise in presence of nanoparticles in tumor. The novelty of this method is the magnetic moments achieved at varying proportions of particles at the Curie temperature of the nanoparticles are specific to generate heating effect(hyperthermia) and visible magnetization or T1 relaxation constants (for MRI) or Hounsfield Number(for X-Ray).

Newer Concepts of Tumor Inflammation and Hyperthermia:
In initial stage of tumor, cells experience the events of hypoxia (low oxygen and low oxidative phosphorylation); progressive inflammation (cytokine synergy); heat shock protein element(gene) trigger to synthesize cAMP mediated heat shock proteins with lysosomal stimulation (cathespins in pro-apoptosis). Advanced stage of tumor shows high glycolysis; high oxygen/ATP demand; elevated cytokines, interleukines, however, subsequent recovery by lysosomal enzymes (towards normal) leads to less tumor cell death and high tumor vascularization (low inflammation, less energy demand and normal temperature). We postulate that hyperthermia may cause supply of enthalpy heat (activation energy) to lysosomal en-
zymes at normal rate; keeping normal cytokines and humoral immunity as most important event.

The heating power area under loop enclosed by hysteresis was calculated by heating power dissipated by particles in magnetic field, \( H \) as following:

\[
P = f \cdot M \cdot dH
\]  

(1)

where \( f \) is frequency of AC magnetic field and \( M \cdot dH \) is hysteresis loop area as shown in Figure 3.

2. MATERIALS AND METHODS

The batch method of co-precipitation was adopted as previously described by Saleh et al.[12] The particles in the form of complexes were synthesized by chemical co-precipitation method. We present here a model \( Zn_{x}Gd_{y}Mn_{1-x}Fe_{3}O_{4} \) chemical co-precipitation method using different Gd proportions \( x \) such as \( Mn_{0.5}Zn_{0.5}GdxFe_{(2-x)}O_{4} \). In our lab, the following method was standardized as:

(1) 0.1 M solution of the metal salts \( FeCl_{3}, Fe_{2}SO_{4}, ZnSO_{4} \) and \( GdCl_{3} \)

(2) Added to an 8 M solution of NaOH.

(3) The mixture was stirred vigorously at 90 ºC for 40 minutes.

(4) The synthesized Zn Gd Fe nanoparticles filtered with Size up to 10 nm.

(5) Washed 3 times with distilled water and 3 times with acetone.

(6) The particles allowed to dry in nitrogen gas at room temperature.

2.1. Polymer PEG Encapsulation for Nanospheres

A following batch process was developed using above steps for preparing composite particles as described initially elsewhere [12]. Polyethylene glycol PEG was used to encapsulate Mn, Zn, Fe and Zn, Gd, Fe nanoparticles to improve their biocompatibility. Encapsulation of Mn, Zn, Fe and Zn, Gd, Fe nanoparticles was performed using polymer emulsion method patented by modified solvent evaporation method. The ingredients were used: Polyethylene glycol (PEG) MW: 1,540; 2 gm (polymer), Methylene Chloride: 10 ml, 13.2 gm (solvent), Water: 40 ml (Aqueous medium), Sodium dodecyl sulphate: 0.33 gm (Emulsifying agent), 1-Octanol: 1.1 ml, 1.32 gm (Inhibitor compound) and Mn, Zn, Fe and Zn, Gd, Fe particles: 50 mg. The magnetic particle: polymer ratio was approximately 1:40. The sodium dodecyl sulphate and 1-octanol were dissolved in 40 ml of distilled water using a magnetic stirrer. Later 50 mg of Mn, Zn, Fe / Zn, Gd, Fe was added. The polymer phase was prepared by dissolving 2 gm of PEG into 10 ml of methylene chloride. A crude emulsion was formed by adding the polymer phase to the aqueous medium phase. It was sonicated using an ultrasonicator 5 times in steps of 3 minutes and stirred inside a round bottom flask for 12 hours at 700 rpm. The solvent was then removed using vacuum evaporation method. The polymer encapsulated particles formed were washed with acetone and stored under PBS buffer solution.

These Mn-Zn-ferrite particles and Gd substituted Mn-Zn-Ferrite particles were obtained via chemical co-precipitation and ferritization. First the metal salts were co-precipitated into hydroxides. This was done by addition of aqueous solution of metal salts in water to the co precipitating base (e.g. NaOH, CH₃NH₃OH etc.). For the case of Mn-Zn Ferrite particles the reaction occurs as follows:

\[
(1-x)Mn^{2+} + xZn^{2+} + 2Fe^{3+} + 8OH^{-} \rightarrow (1-x)Mn(OH)_{2}.xZn(OH)_{2}.2Fe(OH)_{3}
\]

Soon after, this precipitate was transformed into ferrite by heating it in the precipitation alkaline solution (ferritization). The reaction for Mn-Zn ferrite particles was as follows:

\[
(1-x)Mn(OH)_{2}.xZn(OH)_{2}.Fe(OH)_{3} \rightarrow Mn_{1-x}Zn_{x}Fe_{2}O_{4}.nH_{2}O + (4-n) H_{2}O
\]

2.2. Measurement of Reaxation Constants in Nanometals and Tumor Tissues

The measurement of inverse longitudinal \((1/T1)\) and inverse transverse relaxation \((1/T2)\) constants of nanometals in solutions and tumor excised tissues was performed on Bruker-Spec 60 desktop model as described elsewhere [15]. Different chemicals \( FeCl_{3}.6H_{2}O, GdCl_{3}.6H_{2}O, MnCl_{2}.4H_{2}O \) and \( ZnSO_{4}.7H_{2}O \) were used to obtain \( Fe^{3+}, Gd^{3+}, Mn^{2+} \) and \( Zn^{2+} \) ions in the aqueous solution. This salt solution at 90 ºC was added to 8M NaOH solution at 90 ºC followed by vigorous stirring. The stirring and heating at 90 ºC was continued for a minimum of 40 mins. It has been previously reported elsewhere that heating for over 40 mins does not produce any significant changes in the particles properties [17-19]. The product was then filtered, washed with distilled water and finally washed and dried with acetone.

The hysteresis curves were obtained at room temperature using a vibration sample magnetometer (VSM). A Quantum Design SQUID was used to study the temperature dependence of the magnetization [19].

Figure 3. A representative hysteresis loop is shown with different regions of the loop.
3. RESULTS

All the samples were examined by Transmission Electron Microscopy (TEM), X-ray powder diffraction (XRD). The Gd Zn-ferrite nanoparticles and Gd Zn-ferrite HSA 20 nm sized encapsulated nanoparticles in ethanol were deposited over a Cu grid as shown by TEM in Figure 4.

Gd Zn-ferrite nanoparticles and Gd-XRD diagrams for the samples are shown in Figure 4. All major peaks were indexed to the standard pattern for Mn-Zn Ferrite.

3.1. Hysteresis Curves

The hysteresis curves at room temperature for the samples with Gd (x = 0, 0.2, 0.5, 0.7, 1.0, 1.5) were obtained using a vibration sample magnetometer shown in Figure 6, by subjecting them to a field in the range of 0 to 5,000 G as shown in Figure 6. All these samples were observed as soft-magnetic with specific hysteresis loops as shown in Figure 7 where hysteresis curve is proportional to the energy dissipated in the form of power heating loss.

3.2. Saturation Magnetization

The saturation magnetization behavior was specific to gadolinium proportion and temperature dependent. It showed maximum value at gadolinium proportion 0.5 and Curie temperature 412 K as shown in Table 1.

3.3. Temperature Dependence of Magnetization

Table 1. The table shows the variation in saturation magnetization and Curie temperature with increasing Gd proportion.

<table>
<thead>
<tr>
<th>Gadolinium proportion (x)</th>
<th>Curie Temperature (K)</th>
<th>Saturation magnetization (EMU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>320</td>
<td>20</td>
</tr>
<tr>
<td>0.2</td>
<td>409</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>412</td>
<td>29</td>
</tr>
<tr>
<td>0.7</td>
<td>406</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>414</td>
<td>24</td>
</tr>
<tr>
<td>1.5</td>
<td>382</td>
<td>9.5</td>
</tr>
</tbody>
</table>
Figure 7. The hysteresis curves are shown at different temperatures of 28 °C, 37 °C, 47 °C. It can be seen from the figure that the remnant magnetization $\sigma$, the saturation magnetization $M_s$ and the area of hysteresis loop were found to be decreasing with increasing temperature, e.g., the power loss decreases with increasing temperature. (ASME IMEC Seattle meeting 2008: Presenter: Dr CJ Chen IMECE2007-43617).

Figure 8. The temperature and magnetic moment relationship is shown for samples with different Gd proportions in the sample. Note the moments of nanoparticles initially rise at lower range of temperatures and saturation magnetization shows minimal variation at Curie temperature on right side of the curves (ASME IMEC Seattle meeting 2008: Presenter: Dr CJ Chen IMECE2007-43617).

Figure 9. The figure shows variation of Curie temperature at different gadolinium proportion in the magnetic particle samples (ASME IMEC Seattle meeting 2008: Presenter: Dr CJ Chen IMECE2007-43617).

Table 2. The Table shows specific T1 relaxivities of these different nanometal sample concentrations at different temperatures in the range of 41°C specifically near to the Curie temperature.

<table>
<thead>
<tr>
<th>Concentration (in μg/ml)</th>
<th>Temperature (°C)</th>
<th>1/T2 (ms)</th>
<th>1/T1 (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>41</td>
<td>0.264</td>
<td>0.168</td>
</tr>
<tr>
<td>400</td>
<td>0.283</td>
<td>0.154</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.324</td>
<td>0.148</td>
<td></td>
</tr>
<tr>
<td>Gadolinium:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>41</td>
<td>0.298</td>
<td>0.151</td>
</tr>
<tr>
<td>400</td>
<td>0.362</td>
<td>0.136</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.284</td>
<td>0.153</td>
<td></td>
</tr>
<tr>
<td>Zinc:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>41</td>
<td>0.375</td>
<td>0.134</td>
</tr>
<tr>
<td>400</td>
<td>0.286</td>
<td>0.152</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.304</td>
<td>0.150</td>
<td></td>
</tr>
<tr>
<td>Lanthanum:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>41</td>
<td>0.274</td>
<td>0.156</td>
</tr>
<tr>
<td>400</td>
<td>0.293</td>
<td>0.152</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.376</td>
<td>0.133</td>
<td></td>
</tr>
<tr>
<td>Gold:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>41</td>
<td>0.276</td>
<td>0.138</td>
</tr>
<tr>
<td>400</td>
<td>0.285</td>
<td>0.151</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.385</td>
<td>0.144</td>
<td></td>
</tr>
</tbody>
</table>

The temperature dependence of magnetization was observed by using a Quantum Design SQUID. For this a constant field of 100G was applied and the moment was measured by varying the temperature from 0 K to 450 K. Figure 8 shows the superimposed temperature dependence on Magnetization plots for all the samples. The Curie temperature was calculated by extrapolation of the linear sections of the temperature dependence plots [18]. The variation in Curie temperature with increasing Gd proportion is plotted in Figure 9.

3.4. Proton NMR peaks and Relaxation Constants of Nanoparticles

Magnetic particles Manganese (Mn), Zinc (Zn), Gadolinium(Gd), Gold(Au) and Iron(Fe) with proportion ($x=0.5$) in the samples at different concentrations of 200, 400, 1000 μg/ml were tested at different temperatures of 41°C. The NMR peak were used to calculated $1/T1$ and $1/T2$ as
Table 3. Emerging thermal mapping hybrid applications of thermal mapping and hyperthermia monitoring. Different techniques of temperature mapping with hyperthermia monitoring are shown with asterisk for their routine use; research use; and in infancy status in use.

<table>
<thead>
<tr>
<th>Hybrid Modality of thermal mapping with hyperthermia</th>
<th>Physical property for thermal mapping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasound-MR*</td>
<td>Echo and moment</td>
</tr>
<tr>
<td>X-ray and MRI*</td>
<td>Attenuation/moment</td>
</tr>
<tr>
<td>Optical and MRI**</td>
<td>Ligand and moment</td>
</tr>
<tr>
<td>PET and CT**</td>
<td>SUV and attenuation</td>
</tr>
<tr>
<td>PET and MRI**</td>
<td>SUV and moment</td>
</tr>
<tr>
<td>Optical/Molecular imaging***</td>
<td>Ligand specificity</td>
</tr>
</tbody>
</table>

shown in Table 2. These peak patterns also indicated the reasonable limit of temperature between 40-42°C as optimal temperature range for T1 measurement. The concentration of magnetic particles in the range of 200-400 µg/ml and below 1000 µg/ml was sufficient to measure T1 relaxation constants of particles.

4. DISCUSSION

In this study, Gd, Mn, Zn, ferrites with various Gd proportions were analyzed to study the effect on the magnetic properties of these particles and also to find a combination which will result in particles having a Curie temperature suitable for hyperthermia application. TEM suggested the size of these particles in the range of 20 nm and polymer PEG encapsulated nanospheres were measured in the range of 50-70 nm. The particles were distinct by XRD peak patterns of zinc, Gd and iron oxide suggesting their distinct identity after PEG encapsulation in the nanospheres. In the following section different debated views are displayed for tumor temperature rise by magnetic nanoparticles Mn, Zn, Gd, Au, La, Fe usable by multimodal imaging techniques.

A simplistic approach of tumor cell metabolic integrity loss associated with ATP loss, energy depletion, hypoxia leading to inflammation by lysosomal enzyme stimulation and cytokine synergy was assumed as pro-inflammatory cytokines and nitric oxide (NO) responsible for tumor cell killing. Under the condition of hyperthermia, the expression of pro-inflammatory cytokine (e.g. IL-6) and inhibitory (anti-inflammatory) cytokine (IL-10) were regulated. These temperature-dependent changes in the expression of IL-10 may imply an important clinical marker for hyperthermia-related tumor cell killing [28]. However, the mechanistic relation among temperature rise in tumor cells, energy depletion, and inflammation is still unclear. Heat shock treatment, inducing heat shock protein synthesis, also affected the regulation of cytosolic IκB and translocation of NF-κB into the nucleus [29].

The hysteresis curves showed sigmoid behavior of these nanoparticles and its magnitude dependent on temperature rise till temperature of particles reaches Curie temperature during AC vibrating resonator application. It suggests the hysteresis as major contributor at peculiar temperature end point so called “Curie temperature” in heating up to 70% of total heat as described in said section “Heat calculation of hyperthermia”. The similar behavior was reported earlier without much information of contribution in heating effect of tumors [15].

It was observed that the saturation magnetization of the particles drop with increasing Gd proportion. The initial increase in the saturation magnetization can be explained by considering that the Gd³⁺ ions have a large spin magnetic moment per atom (7µB) as compared to that of Fe²⁺ ion (5 µB) [20-23]. Addition of Gd³⁺ ions results in their occupancy of the octahedral sites. The preference for octahedral sites maybe attributed to their large ionic radii. Since the ionic radii of the Gd³⁺ ions are large, there is a decrease in the distance between these and the oxygen ions when adding Gd ions [24]. As a result the ions at the octahedral sites no longer have their moments parallel to each other.

A part of these ions have moments aligned antiparallel to the other atoms on these octahedral sites. This results in a reduction in the net magnetic moment of the octahedral atoms. As the Gd substitution is increased, more and more octahedral atoms have their moments as antiparallel. As a result the saturation magnetization drops. Upadhyay et al. [23] have synthesized Gd substituted Mn-Zn Ferrite nanoparticles using chemical coprecipitation. They observed an increase in the pyromagnetic co-efficient (HTM) of the resultant particles. The increase in the pyromagnetic co-efficient is desirable because it results in a steeper slope of the magnetization vs temperature plot which in turn ensures that the magnetization decreases rapidly as the temperature approaches the Curie temperature. This rapid decrease in magnetization means that the particles are heated up faster at temperatures below the Curie temperature and suddenly stop being heated near the Curie temperature which is a desirable property for Hyperthermia application. From Fig. 5 it can be seen that there is an increase in hyperthermia monitoring. Different nanoparticle composites are shown with their use in thermal mapping technique and possible use in hyperthermia monitoring. The potential nanoparticles in hyperthermia use are shown with plus sign for routine use, research use ++, and in infancy state + or not established – at present.

Table 4. Potential newer nanoparticles used in thermal mapping and hyperthermia monitoring. Different nanoparticle composites are shown with their use in thermal mapping technique and possible use in hyperthermia monitoring. The potential nanoparticles in hyperthermia use are shown with plus sign for routine use ++, research use +++, and in infancy state + or not established – at present.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Thermal mapping</th>
<th>Hyperthermia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc-Gadolinium-Ferrite</td>
<td>MRI, CT</td>
<td>+</td>
</tr>
<tr>
<td>Gold-Ferrite</td>
<td>MRI, CT</td>
<td>++</td>
</tr>
<tr>
<td>Gadolinium-Ferrite</td>
<td>MRI, CT</td>
<td>+++</td>
</tr>
<tr>
<td>Lanthanum-Ferrite</td>
<td>MRI</td>
<td>+</td>
</tr>
<tr>
<td>Calcium</td>
<td>Optical, Molecular</td>
<td>+</td>
</tr>
<tr>
<td>Gd-Mn-Zn-Ferrite</td>
<td>MRI, CT</td>
<td>+++</td>
</tr>
<tr>
<td>Mn-Gd-Ce</td>
<td>MRI</td>
<td>--</td>
</tr>
<tr>
<td>La-Sr-Mn</td>
<td>MRI</td>
<td>--</td>
</tr>
<tr>
<td>Nanoparticle agents</td>
<td>MRI</td>
<td>--</td>
</tr>
</tbody>
</table>
in Curie temperature with Gd substitution. Magnetic nanoparticles have found utility in many biological applications, including imaging, cancer therapy, drug delivery, sensing and hyperthermia for tumor therapy. In general, hyperthermia raises the tissue temperature between 41.5 - 46 degrees Celsius to kill cancerous cells while preserving the normal cells. Several nanoparticles such as gold, zinc, gadolinium, lanthanum, and calcium have emerged as potential hyperthermia agents. Recently, new composite materials such as Mn-Zn-Fe, Co-Gd-Zn and Zn-Gd-Fe nanoparticles with stable magnetic behavior have replaced magnetic oxides for use in hyperthermia at our lab. These composites generated sufficient heat at room temperature and stop heating at the Curie temperature $T_c$ of the respective nanoparticle system.

Gold nanoparticles (AuNP) killing the cancer cells was first reported by Hainfeld [98]. However, after injecting gold particles in animals and irradiation them by 250 kV, X-rays caused tumor shrinkage and enhanced survival rate by four fold. The major challenge was localization of gold particles because of vascular leakage in the tumor but maximized particles entry in the tumor. Still, there are ample potential evidences in favor of gold enhanced x-ray hyperthermia in tumor treatment by killing. The application of gold particles in nanomedicine is its promise in radiotherapy of cancer. The Au-198 ($\beta_{\max} = 0.96 \text{ MeV}; \tau/2 = 2.7 \text{ days}$) and Au-199 ($\beta_{\max} = 0.46 \text{ MeV}; \tau/2 = 3.14 \text{ days}$) make them suitable in radiotherapy. In addition, gold particles display gamma emissions for dosimetry and pharmacokinetic studies.  Therapeutic agents derived from gold nanoparticles provide a higher radioactivity dose to tumor sites. Furthermore, tumor-specific nanotherapeutic agents as a nanoparticle while tagged with peptides selective to receptors and over-expressed by tumor concentration offer another advantage. The gold nanoradioisotopes encapsulated within a nanocomposite device were reported as vehicles to transport radioactive particles to tumor sites. In this approach, particle size and number play a significant role such as nanocomposites made of monodisperse hybrid radioactive gold nanoparticles immobilized by dendritic polyamidoamine matrix prepared by reaction of polymer and tetrachloroaurate HAuCl$_4$ solution. The salt formation between these solutions ensured the effective encapsulation of gold within the matrix using neutron irradiation in mice B 16 melanoma, prostate DU 145, human KB squamous cell carcinoma xenograft models. The property of polymer with $\beta$ emitting Au-198 enriched nano-device proved useful in tumor therapy. Moreover, the polymer enhances the stealthiness of magnetic nanoparticles by preventing macrophage recognition of particles as less toxic and resisting oxidation to make them valuable in multifunctional hyperthermia and imaging modalities. Another issue in tumor treatment is delivery of chemotherapeutic agents within gold nanoparticles. It becomes effective as a tumor killing and targeted delivery tool.

These multicompont particles are made of Zn$_x$Mn$_{1-x}$Fe$_2$O$_4$ and Mn$_x$Zn$_{1-x}$Fe$_2$O$_4$ composites synthesized by physical and chemical co-precipitation methods. These particles displayed the increased tissue temperature and hyperthermia nature. Additionally particles in the form of Ni$_{1-x}$Cr$_x$ were also formed. These particles may be encapsulated in thermo-sensitive polymer that dissolves when melted. The magnetic Mn-Zn ferrite and Gd substituted Mn-Zn ferrite particles synthesized by the chemical co-precipitation method exhibited a specific behavior applicable in hyperthermia.

Paramagnetic gadolinium offers the excellent detection limit using contrast injection of 15 gm/gm tissue in clinical imaging. In our lab, these particles were encapsulated in thermosensitive polymer that dissolves after melting. These nanosized particles exhibited specific behavior of magnetic moments at Curie temperature and provide a window to evaluate their heating effect in tissue. Addition of Gd$^{3+}$ ions up to proportions of $x=0.5$ results in an increase in the net moment. Further addition of the Gd$^{3+}$ ions results in a decrease in the net moment or saturation magnetization (SM). To prove it, the nanoparticle complex Mn-Gd-Ce was characterized by placing particles in a tube and its temperature increase with time as shown in Figure 9. The SM increases as temperature rises close to Curie temperature initially and subsequently SM decreases at temperature higher than the Curie temperature at increasing proportion of Gadolinium. Recently, nanosize Gd substituted Mn-Zn ferrite particles have been synthesized by a chemical coprecipitation method. These particles were mostly soft-magnetic. Gd substituted Mn-Zn Ferrite nanoparticles using chemical co-precipitation demonstrate an increase in the pyromagnetic co-efficient (HTM)/(GME) of the resultant particles. The increase in the pyromagnetic coefficient is desirable because it results in a steeper slope of the magnetization v/s temperature plot which in turn ensures that the magnetization decreases rapidly as the temperature approaches the Curie temperature.

The Silica-Coated Lanthanum-Strontium Manganite Particles were prepared suited for hyperthermia. The core-comprising LaSr$_x$-manganites with different stoichiometries, ranging from La$_{0.5}$Sr$_{0.5}$MnO$_3$ to LaMnO$_3$, were synthesized as silica-coated magnetic particles with designable Curie temperature, offering a wide range of possibilities of adapting the material to practical instrumental setups in drug delivery and hyperthermia treatments. The relationship was based on temperature dependence of the proton chemical shift of water $\leq 0.01 \text{ ppm}/^\circ \text{C}$ at $\leq 0.7 \text{ Hz}$ at 1.5T for 1 $^\circ$C temperature change using lanthamide complexes.

Malignant Hyperthermia (MH) is a hypermetabolic syndrome that results from the altered control of sarcoplasmic reticulum (SR) Ca$^{2+}$ release. Recent study established the imaging of cytosolic [Ca$^{2+}$]$^{\text{c}}$ ([Ca$^{2+}$]$^{\text{c}}$) in single cells grown from human skeletal muscle biopsies and in H9c2 myotubes in response to a low dose of halothane on the cells derived from MH susceptible patients. The
Ca²⁺ imaging in single cells is a promising candidate for the development of a new diagnostic and hyperthermia procedure of MI.

Recent improvements in optical imaging techniques now allow these microdomains to be visualized as single channel calcium fluorescence transients (SCCaFTs), providing information about channel properties to monitor the activity and localization of microdomain calcium complexes. Other emerging possibilities are MR molecular imaging by gadolinium based perfluorocarbons, integrins, fibrins, and monoclonal antibody labeled nanoparticles. Recently remarkable progress is reported in real-time monitoring of thermal therapy and ablation using multimodal imaging techniques.

The emergence of a new class of nanoparticles as contrast agents have created the desire of localized tumor molecular imaging based monitoring of tumor hyperthermia and molecular mapping responsible for thermal heating of a tumor as represented in Table 4. However, multimodal and multifunctional approaches of hyperthermia monitoring and thermal mapping are still in their infancy to use them in routine. The present growing interest of thermal mapping and hyperthermia monitoring is to achieve a rapid thermotherapy heating effect over focused tumor areas accurately by molecular imaging techniques.

Regional hyperthermia in combination with chemotherapy or/and radiotherapy has become state of the art as effective hyperthermia for locally advanced deep-seated tumors as shown in Table 3. The thermometry using 3-dimensional US-MR-hyperthermia hybrid-system was installed and tested in phantoms and under clinical conditions in patients. The simultaneous MR-imaging and T1-relaxometry at 0.2 Tesla during RF heating was performed using T1 sensitive pulse sequences to serve a basis for non-invasive MR-thermometry. The subtraction of T1 parameter maps before and during heating visualized the changes in T1. The patterns of T1 relaxation changes during hyperthermia treatment may prove to be useful for spatially resolved thermometry and thus help improve the hyperthermia therapy.

5. CONCLUSION

Nanosize Mn-Zn ferrite and Gd substituted Mn-Zn ferrite particles have been synthesized by chemical co-precipitation method. The samples are observed to be soft-magnetic. Addition of Gd³⁺ ions up to proportions of x=0.5 results in an increase in the net moment. Further addition of the Gd³⁺ ions results in a decrease in the net moment. The saturation magnetization increases then decreases with increasing proportion of Gadolinium. The Curie temperature increases with addition of Gadolinium. But addition of Gd in proportions more than x=1.0 results in a decrease in Curie temperature. Mn₀.₅₉₁₆Zn₀.₅₄₇Ferrite ferromagnetic nanomagnetic particles showed sharp ferromagnetic to paramagnetic transition behavior at specific temperature.

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Fabrication and characterization of HAp/Al₂O₃ composite coating on titanium substrate

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ABSTRACT

HAp/Al₂O₃ composite coating was fabricated onto micro-arc oxidized titanium substrate using a combination of electrophoretic deposition and reaction bonding process. SEM, EDS and XRD were employed to characterize the titanium substrate and as-prepared coatings. The interfacial bonding strength of the sintered composite coating was tested by shear strength testing experiment. Results show that the green form composite coating can be easily sintered with no cracks and decomposition at 850°C, the bonding strength to the substrate is significantly improved compared with the single HAp coating.

Keywords: Hydroxyapatite; Composite coating; Electrophoretic deposition; Reaction bonding process

1. INTRODUCTION

Titanium and its alloys are widely used for dental and orthopedic implants, because of their high mechanical properties, chemical stability, and biocompatibility. Due to its poor osteoconductive properties, coating of bioactive hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂, HAp), which has similar chemical and crystallographic structure to the main inorganic phase of human bone tissues, onto biomedical titanium implants has attracted widespread interest in the orthopaedics biomedical field [1].

Many techniques have been investigated for depositing HAp onto metallic implants, including plasma spray [2], thermal spray [3], sol-gel processing [4], electrolytic deposition [5] and electrophoretic deposition [6]. Among these techniques, plasma spraying is the most developed process and has been used in clinical practice; however, this process suffers from facts that it requires complex and costly equipment and being a line-of-sight process which is difficult to apply uniform coatings on implants with complex geometries.

Electrophoretic deposition (EPD) is a colloidal forming technique where charged, colloidal particles in a stable suspension are deposited onto a positively charged substrate by the application of electric field [7]. EPD has recently gained increasing interest in the processing of advanced ceramic materials and coatings not only because of its coast-effectiveness requiring simple apparatus, but also it offers important advantages in the deposition on substrates of complex geometry [8]. As for many ambient-temperature powder coating processes, the deposit is in the form of a loosely packed particles which must be subsequently densified by heating the coated implant to elevated temperatures [9]. Electrophoretic deposition of HAp coating onto metallic substrate has gained wide interest and previous researches have demonstrated that EPD is an attractive method for formation of biomedical implants and a number of advantages of this method have been suggested [10]. However, most of the reports demonstrated that bonding strength between HAp coating and titanium substrate is commonly low and far from the requirement for clinical application.

The main problem associated with the EPD process is the difficulty in the sintering of the coatings. First, high sintering temperature is required for full densification of the green coatings [9]. Lower sintering temperature leads to weakly bonded and lowly-densified coatings, whereas higher temperature can result in degradation of the metal substrate and decomposition of HAp coating. Decomposition of the HAp coating is undesirable as it leads to an enhanced in vivo dissolution rate. Sintering temperatures ideally should be below 1000°C [9]. Second, the thermal expansion coefficient of titanium substrate is much lower than that of HAp (αₜ₀= 8.7×10⁻⁶/K, αHAp=13.6×10⁻⁶/K), so large thermal contraction mismatch would arise and tend to induce the formation of cracks when cooled from the elevated temperatures; besides, a significant firing shrinkage during sintering will lead to the formation of cracks in coatings as well.

In the present work, reaction bonding Al₂O₃ with relatively lower thermal expansion coefficient (αAl₂O₃=8.3×10⁻⁶/K) was introduced into the HAp coating to shorten the thermal expansion coefficient difference with the titanium substrate. Meanwhile, the reaction bonding process would overcome problems caused by the firing shrinkage during sintering [7]. Both the two advantages have been proved to be beneficial in avoiding the formation of cracks and improvement of bonding strength of ceramic coatings [11].


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Surface modification of the titanium substrate was also concerned. Chemically stable TiO$_2$ can act as a bonding layer and chemical barrier, which improves the interfacial bonding, and prevent in vivo release of metal ions [12]. Among the methods used to produce the oxide layer on titanium substrate, the micro-arc oxidation (MAO) method has gained much interest, where anticorrosive and rough oxide coatings can be easily fabricated [13-14]. Rough morphology has been proved to be beneficial in mechanically anchoring the as-deposited coating [15]. So MAO surface modification was done prior to the deposition of composite coating.

2. EXPERIMENTAL

Commercially available titanium plates which were shaped in a size of 30mm×10mm×0.8mm, were used as the substrate materials. All the specimens were mechanically polished with SiC water-proof abrasive papers. Then they were degreased in a certain base solution and pickled in an acid solution containing 100 mL/L HF and 300 mL/L HNO$_3$. After that, the specimens were treated with MAO in a sulfate solution. A platinum plate was used as the counter electrode.

For preparation of the EPD suspension, HAp and Al particles with an average particle size of 0.5 μm were dried previously and dispersed in absolute ethanol. Drops of nitrate were used to adjust the pH value of the suspension to 4-5 approximately. Then the suspension was stirred in an ultrasonic agitator for 1 hour and aged for 1 day to allow full charging of the particles dispersed. Prior to the deposition, the suspension was again stirred in the ultrasonic agitator for 30 min. EPD was carried out using applied voltages in the range 30-40 V for 30 s. Two parallel stainless steel plates were used as the anodes. The deposited specimens were dried in air and then stored in a drying container.

The heat treatment was done in a tubular electric resistance furnace. The furnace was heated at a rate of 5°C/min to 660°C and held at this temperature for 2 hours to allow the occurrences of melting and oxidation of Al particles. The temperature was then increased at 5°C/min to 850°C or 900°C and held for 2 hours for sintering. At last, the furnace was cooled in a rate of 1°C/min to room temperature. During the treatment, Ar atmosphere of high purity was controlled in a proper velocity to flow through the tube to protect the substrates from excessive oxidation in elevated temperatures.

The surface and cross-sectional morphologies of the MAO titanium and as-prepared coatings was observed by LEO1530-FESEM (Germany). The element composition of the composite coating was analyzed through EDS (Oxford, England) attached to the FESEM. The phase composition and thermal stability of the composite coating were analyzed by X-ray diffraction (Panalytical X’pert, Philips).

The interfacial bonding strength was tested by shear strength testing experiment according to ASTM-F1044 standard. This test relies on a bonding agent to remove the coating with applied shear force as shown in Figure 1. The value of the bonding strength can be calculated from the fracture force over the stressed area.

3. RESULTS AND DISCUSSION

Many researches have demonstrated that surface modify-
Figure 4. SEM images of the surface morphology of HAp/Al2O3 composite coating sintered at 850°C. (a: ×100; b: ×20,000).

Figure 5. SEM images of the surface morphology of single HAp coating sintered at 850°C. (a: ×100; b: ×20,000).

Figure 6. Cross-sectional morphology of HAp/Al2O3 composite coating sintered at 850°C.

Figure 7. XRD patterns of HAp/Al2O3 composite coating. (▼: TiO2 (Rutile); ■: Cubic-Al2O3; □: Al; ▽: Decomposition products; The others: HAp)
cation is essential to guarantee coating adhesion to metallic substrate [14]. MAO is an advance coating process for forming oxide layer on some anodic metal substrate which is accompanied by visible plasma-like sparking at the anode surface [13]. Prior to EPD of the composite coating, the titanium substrate was treated by MAO in a sulfate solution under constant current density of 50 mA/cm². Surface morphology in Figure 2 shows that the oxide film fabricated is rough and porous, which has been proved to be beneficial for mechanically anchoring the as-deposited coating [15].

The green form of HAp/Al composite coating was co-deposited onto the titanium substrate from a suspension containing 10 g/L HAp and 10 g/L Al particles using an applied voltage of 30 V for 30 s. The chemical compositions of the composite coating were determined through EDS element analysis. Relating spectrum is shown in Figure 3. The results show that elements Al, Ca, P and O are all present in the composite coating, which confirms that the co-deposition of HAp and Al under the present condition is feasible. Quantitative analysis based on the spectrum shows that the weight percentage of Al in the composite coating was about 17.5%, and the Ca/P mole ratio was about 1.70, which is approximately equal to the stoichiometric ratio of HAp, and this confirms the chemical stability of HAp in the suspension.

Figure 4 shows the surface morphology of the as-prepared HAp/Al₂O₃ composite coating after sintered at 850°C. The surface morphology of single HAp coating fabricated under the same conditions is also shown in Figure 5 as comparison. As shown in Figure 4 (a), no cracks were observed from the surface of the composite coating; while for the single HAp coating as shown in Figure 5 (a), numerous cracks were found because of the firing shrinkage during sintering. Figure 4 (b) shows that the composite coating was well sintered. It is obvious that particles in the composite coating bond with each other and grain size grows; however, for the single HAp coating shown in Figure 5 (b), most particles appear to remain stand-alone, bonding among the particles is not as full as in the composite coating and there is nearly no growth of grain size. It can be excluded that the sintering property is greatly improved by the addition of Al to the green form coating. The irregular shape of grains in the composite coating implies the presence of liquid phase during sintering which is known to be beneficial in promoting the mass transport and bonding among grains. The volume expansion associated with the oxidation reaction of Al→Al₂O₃ partially compensates for the sintering shrinkage and prevents the formation of cracks.

Figure 6 shows the cross-sectional morphology of the composite coating after sintered at 850°C, which presents an impression of a layered interfacial structure. A dense oxide layer, which came from a composite oxidation of MAO and heating oxidation, is present between the composite coating and substrate. The oxide layer acts as a bonding layer to bond the composite coating and substrate together, which is able to improve the interfacial bonding strength. Besides, the dense oxide layer is beneficial in preventing the ion release from the metallic substrate [13].

Figure 7 shows XRD patterns of the sintered coatings. All the diffraction patterns confirm the presence of rutile-TiO₂ as the inner bonding layer shown in Figure 6, and HAp as the main phase in the composite coating. The sharp and clear reflections corresponding to HAp confirm the phase purity and high crystallinity which is critical for in vivo stability of the implants. The confirmation of the existence of Al₂O₃ in the HAp coating puts the lowering the thermal expansion coefficient of the composite coating into effect and contributes to the increase of interfacial bonding strength.

The thermal stability of the as-prepared composite coating was also studied to determine a proper heat treatment condition. At the sintering temperature of 850°C, no signs of HAp decomposition can be found in the relating XRD pattern. While sintered up to 900°C, several peaks (marked by “\^\wedge\”) of new phase arise in the corresponding XRD pattern and imply the thermal decomposition of HAp phase; but reflections characteristic for HAp still well match its reference pattern, which suggests that decomposition degree of HAp is not serious. Hence, in order to guarantee the chemical and structural integrity of HAp and its in vivo properties, the sintering temperature should necessarily be controlled to be below 900°C.

Interfacial bonding strength of the as-prepared HAp/Al₂O₃ composite coating was tested. Results are shown in Tab.1 where each value represents a statistic average of three test data. Obviously the bonding strength of HAp/Al₂O₃ composite coating is commonly higher than that of single HAp coating under the two sintering temperature. The improvement of interfacial bonding can mainly be attributed to the application of reaction bonding process. The process overcome the cracking problem and improved the sintering property of the coating as analyzed above. On the other hand, the formation of reaction bonding Al₂O₃ with relatively lower thermal expansion coefficient shortens the thermal expansion coefficient difference between the coating and the titanium substrate, and improves the interfacial bonding as well.

4. CONCLUSION

Rough and porous oxide film, which has been proved to be beneficial in mechanically anchoring the as-deposited coating, was fabricated on titanium substrate through the MAO technique. The electrophoretic co-deposition of HAp and Al powder was achieved successfully to form HAp/Al composite coating. The sintering temperature of the composite coating should be controlled to be below 900°C due to the thermal decomposition of HAp phase. A crack-free and adhesive HAp/Al₂O₃ composite coating was then successfully fabricated using the combination of electrophoretic deposition and reaction bonding proc-
ess. The reaction bonding process promotes the coating’s sintering densification and improves the substrate’s oxidation resistance during the heat treatment. In comparison with the single HA coatings, the HA/Al\textsubscript{2}O\textsubscript{3} composite coating exhibits much higher bonding strength.

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Application of modified superposition model to viscoelastic behavior of periodontal ligament

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ABSTRACT

The periodontal ligament (PDL) is a soft biological tissue which shows a strongly nonlinear and time dependent mechanical behavior. Recent experiments on rabbit PDL revealed that the rate of stress relaxation is strain dependent. This nonlinear behavior of PDL cannot be described well by the separable quasi linear viscoelasticity theory which is usually used in tissue biomechanics. Therefore, PDL requires a more general description which considers this nonlinearity and time dependency. The purpose of this study was to model strain dependent stress relaxation behavior of PDL using modified superposition method. It is shown herein that modified superposition method describes viscoelastic nonlinearities well and shows a good compatibility with available experimental PDL data. Hence, the modified superposition model is suggested to describe periodontal ligament data, because it can suitably demonstrate both elastic nonlinearity and strain-dependent stress relaxation behavior of PDL.

Keywords: periodontal ligament, viscoelasticity, modified superposition method, stress relaxation

1. INTRODUCTION

A tooth is secured to the alveolar bone by fibrous connective tissue that is called the periodontal ligament (PDL) and schematically is showed in Figure 1. The human PDL stabilizes the tooth in bone and provides nutritive, proprioceptive and reparative functions [1]. It is composed of collagenous fibers and a gelatinous ground substance including cells and neurovascular tissue [2]. The PDL not only strongly binds the tooth root to the supporting alveolar bone but also absorbs occlusal loads and distributes the resulting stress over the alveolar bone [3]. This causes PDL to play a major role in tooth mobility which is very important in prosthodontic and orthodontic treatment and selection of an optimal force system for orthodontic treatment [4]. The PDL has a determinant influence on tooth instantaneous mobility because of lower stiffness in comparison with surrounding tissues [5], and also long term movement because of bone remodeling [6].

Ligaments display time dependent behavior which is typical of viscoelastic materials [7]. Viscoelastic behavior has been observed and studied in articular cartilage [8, 9], periodontal ligament [10-12], ligament [7]. Formulation for modeling viscoelasticity in nonlinear material was first used for modeling the time dependent behavior in soft tissue by Fung [13] and called it “quasi linear viscoelasticity” (QLV). The QLV theory has been applied for PDL by Natali et al. [14]; Toms et al. [2]. These models are composed of two parts: an instantaneous elastic response (often a hyperelastic strain energy function) and a relaxation function. Relaxation function governs the fading memory of current constitutive state on deformation history [15].

It is known from literature, periodontal ligament is a nonlinear viscoelastic material [16, 17], and stress relaxation behavior is a nonlinear function of strain. The rate of stress relaxation decreases with increasing strain [7]. The behavior in these data cannot be described well using Fung quasi linear method [13], because in this formulation stress relaxation is independent of strain. Therefore, two previously presented QLV model for PDL is discussed:

1) Komatsu et al. [12] studied stress relaxation on PDL at different deformations. Experiments were done on seventeen 4-month-old rabbits. The stress relaxation process was well described by a function (relaxation function) with three exponential decay terms and a constant. On the other hand the experiment showed that stress relaxation is a function of strain too, and $G(t)$ (relaxation function) differs at different strains. Komatsu’s model for relaxation function is as follows:

$$G(t) = 0.39 + 0.09 \exp(-t / 0.35) + 0.13 \exp(-t / 4.12) + 0.39 \exp(-t / 403)$$

In the present study, experimental data obtained by Komatsu et al. [12] has been used to describe stress relaxation process for PDL.
2) Toms et al. [2] studied the quasi linear viscoelastic model and applied to mechanical tests of the human PDL. Transverse sections of cadavric premolars were subjected to relaxation tests. In their study the relaxation function described by using an exponential equation. This model was similar to Komatsu's model.

Many reasonably general constitutive models have been proposed to describe nonlinearly viscoelastic materials. A brief description of these models has been studied by Provenzano et al. [7]. In this paper, we have used the modified superposition (or nonlinear superposition) method to describe nonlinear viscoelasticity of PDL to determine whether this method can model strain dependent stress relaxation behavior of PDL.

2. MATERIAL AND METHODS

2.1. Modified Superposition Method

The single integral formulation of the modified superposition method [18, 19] allows the relaxation function to depend on strain level:

$$\sigma(\varepsilon, t) = \int_0^t E(t - \tau, \varepsilon) \frac{d\varepsilon(\tau)}{d\tau} d\tau$$  \hspace{1cm} (2)

The form of the relaxation modulus will be chosen as a non separable strain-dependent power law:

$$E(\varepsilon, t) = A(\varepsilon) \cdot G(\varepsilon, t)$$  \hspace{1cm} (3)

$$G(\varepsilon, t) = e^B(\varepsilon)$$  \hspace{1cm} (4)

The function $A(\varepsilon)$ represents the initial modulus $E_0$, which can be obtained from a stress–strain curve or isochronal curve describing the nonlinear elastic behavior. $G(\varepsilon, t)$ is the relaxation function which itself is function of strain and time. The function $B(\varepsilon)$ describes the strain-dependent rate of stress relaxation. Substituting a Heaviside function into Eq.(2) results in:

$$\sigma(\varepsilon, t) = E_0 e^{B(\varepsilon) t} = \sigma_0 e^{B(\varepsilon) t}$$  \hspace{1cm} (5)

where $E_0$ and $\sigma_0$ represent isochronal values of the tangent modulus and stress, respectively, and can be functions of strain to account for nonlinearities in the elastic response. In addition, Eq.(5) can take on a more predictive form once relaxation rates over a range of strain values are obtained, the dependence of the rate function $B(\varepsilon)$ as a function of strain is known. Stress–strain or isochronal curves can be used to obtain the initial modulus or stress terms, $A(\varepsilon)$ and a polynomial can be fit to the rate range to obtain the function $B(\varepsilon)$; so mechanical behavior of the tissue can be predicted. Hence, the non separable form of modified superposition is able to represent both the elastic and strain-dependent rate nonlinearities that are experimentally observed.

2.2. Application of Modified Superposition Method to PDL

Modified superposition method Eqs.(2-5) was applied to experimental stress relaxation data from rabbit periodontal ligament [12]. As mentioned above, these data demonstrate that the rate of stress relaxation decreases significantly with increasing tissue strain, this behavior have shown in Figure 10. For rabbit PDL modified superposition method fits the experimental data well for all strain levels: $\varepsilon = 0.078, 0.124, 0.17, 0.215$. $\sigma_0$ values are 0.265, 0.53, 1.19, 1.85, MPa for 0.078, 0.124, 0.17, 0.215 strain respectively. $s_0$ is the stress at the start of stress relaxation test ($t = 1$ sec), this is reasonable if assumed that the stress relaxation have not started up to 1 sec, so it is supposed that $t = 1$ sec is initial time for this method. Strain dependent rate of stress relaxation term or $B(\varepsilon)$ can be seen to decrease in magnitude as strain increase (-0.172, -0.127, -0.102, -0.096 for 0.078, 0.124, 0.17, 0.215 strain, respectively) which shows a nonlinearity in the strain dependent rate of stress relaxation.

The rate function or $B(\varepsilon)$ and $A(\varepsilon)$ is a polynomial which is obtained by curve fitting using commercial software MATLAB 7.0 (The Mathwork Inc., Natick, MA). Curve fitting rate function (Fig 4), and initial modulus (Fig 5) versus strain were done using experimental data from Komatsu et al. [12].

3. RESULTS
Figure 3. Relaxation function with three exponential decay terms and a constant presented by Komatsu [12] under the deformation of 78 μm.

Figure 4. Fitting of polynomial function (curve) to experimental stress relaxation rate of Komatsu et al. [12] for multiple rabbit periodontal ligament at multiple strain levels.

Figure 5. Fitting of power function (curve) to experimental initial modulus of Komatsu et al. [12] for multiple rabbit periodontal ligament at multiple strain levels.

Figure 6. Comparison of experimental data (points) and predicted (line) relaxation function behavior, for strain of 0.215 and initial stress of 1.85 MPa.

Figure 7. Comparison of experimental data (points) and predicted (line) relaxation function behavior, for strain of 0.17 and initial stress of 1.19 MPa.

Figure 8. Comparison of experimental data (points) and predicted (line) relaxation function behavior, for strain of 0.124 and initial stress of 0.53 MPa.

Figure 9. Comparison of experimental data (points) and predicted (line) relaxation function behavior, for strain of 0.078 and initial stress of 0.265 MPa.

Figure 10. Relaxation function at different strain levels, the rate of stress relaxation decreases significantly with increasing tissue strain.
In order to obtain a set of constitutive parameters for PDL, *in vitro* experimental data were analyzed, and the fit of the rate function was done with $R^2$ value of 0.999 for tension relaxation using nonlinear least square method. The results of curve fitting have been depicted in Figure 4. The rate function obtained as follows:

$$B(\varepsilon) = 5.937\varepsilon^3 - 7.299\varepsilon^2 + 2.286\varepsilon - 0.3094$$ (6)

The function $A(\varepsilon)$ was determined by fitting the tangential modulus as a function of strain using nonlinear least square method from typical stress–strain data for the rabbit periodontal ligament with $R^2$ value of 0.967. The results of curve fitting have been depicted in Figure 5. The function $A(\varepsilon)$ obtained as follows:

$$A(\varepsilon) = 42.66\varepsilon^{1.039}$$ (7)

So, the relaxation modulus will take the following form:

$$E(\varepsilon, t) = 42.66\varepsilon^{1.039} \times \frac{5.937\varepsilon^3 - 7.299\varepsilon^2 + 2.286\varepsilon - 0.3094}{t}$$ (8)

Four separate stress relaxation tests at four different strains were then fitted using the predicted rate function. The results for each strain and comparison between the experimental data and the model curve have been demonstrated in Figures 6-9. Figure 10 shows that the rate of stress relaxation decreases significantly with increasing tissue strain which shows the dependence of relaxation function to tissue dilatation. Stress relaxation process for the initial stress of 1.86 MPa and strain of 0.215 which is followed by a gradual reduction up to 600s has been depicted in Figure 10, after 600s the stress decreases approximately to 1.0 MPa and the relaxation function reaches 0.54.

4. DISCUSSION

In this study the ability of modified superposition (nonlinear superposition) method as a nonlinear viscoelastic model has been investigated to describe periodontal ligament behavior which has been experimentally observed. In previous studies, QLV model had been applied for PDL, with QLV theory, the time dependent portion of model $G(t)$ (relaxation function) is independent of strain, however, as experimental data shows the relaxation behavior differs at different strain levels and depends on tissue dilatation too. In this study the time dependent portion of model depends on strain to consider strain history dependence of tissue, too.

The stress relaxation property of PDL was expressed by a non separable strain dependent power law, which showed a good fitting with experimental data under different deformations, but in previous studies stress relaxation property of PDL had been often expressed by exponential decay terms which were functions of time alone, as in the human [2]; rabbit [12]; bovine [20]; pig [14], and strain history dependence of tissue had not been taken into account.

The mechanisms driving viscoelastic behavior in ligament are not yet completely defined. It has been speculated that “the decrease in relaxation rate with increasing strain could be the result of larger strains causing greater water loss (wringing out effect) which causes the tissue to be more elastic (less viscous) than tissues subjected to lower strains” [21]. In other words, at greater deformations because of water loss, the viscous components (matrix) have less influence in tissue mechanics than elastic components, and this causes the tissue to show more elastic properties than viscous.

There are several limitations which must be considered while examining the presented model in this study. In the presented model other effects such as diseased state, effects of age, biochemical changes, temperature, hydration and others have not been considered. The other limitation is related to experimental data, in which collagen fiber bundles in PDL of the specimens are assumed to run almost parallel to the direction of testing.

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Searching maximum quasi-bicliques from protein-protein interaction network

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ABSTRACT

Searching the maximum bicliques or bipartite subgraphs in a graph is a tough question. We proposed a new and efficient method, Searching Quasi-Bicliques (SQB) algorithm, to detect maximum quasi-bicliques from protein-protein interaction network. As a Divide-and-Conquer method, SQB consists of three steps: first, it divides the protein-protein interaction network into a number of Distance-2-Subgraphs; second, by combining top-down and branch-and-bound methods, SQB seeks quasi-bicliques from every Distance-2-Subgraph; third, all the redundant results are removed. We successfully applied our method on the Saccharomyces cerevisiae dataset and obtained 2754 distinct quasi-bicliques.

Keywords: Searching Quasi-Bicliques algorithm; Quasi-biclique; Protein-Protein Interaction Network; Distance-2-Subgraph; Divide-and-Conquer method

1. INTRODUCTION

As high-throughput technologies such as Yeast Two-Hybrid [1] and Affinity Purification/ Mass Spectrometry [2] have made significant progress, human beings have collected a great number of protein-protein interaction datasets. It is meaningful to dig out substrutures from large-scale protein interaction data. Biclique, one kind of the substructures, is common in protein-protein interaction network. Biclique often contains useful biologically meaningful units. For example, the biclique shown in Figure 1 indicates an “all-versus-all” predicted interaction subnetwork [3]. Where most of the edges, each representing a protein-protein interaction, were approved by biological experiments. Furthermore, six proteins on the left side all contain SH3 domain and four proteins on the right side are all with the SH3-binding motifs. Therefore mining biclique can help biologists unveil the cellular function at the molecular level.

However, mining bicliques from graph (or protein-protein interaction network in this study) is a computationally intensive work, and has been proven as NP complete [4, 5, 6]. Although many researchers [7, 8, 9, 10, 11] have developed some algorithms to solve the maximum biclique problem, they often focused on some special characteristics of the graph, so the problem is still intractable. Therefore, in computational biology field, some researchers mined quasi-bicliques instead of exact bicliques. Li [12] used the “frequent pattern” developed by Agrawal [13] to find “all-versus-all subnetwork” (or quasi-biclique). The “existing closed itemset mining algorithms” (proposed by Agrawal [13]) only uses the size constraint on transaction sets to decrease search space, which brings a great number of small maximum bicliques and greatly influences the process speed.

Here, we propose Searching Quasi-Bicliques (SQB) algorithm to detect maximum quasi-bicliques from protein interaction network. By means of Divide-and-Conquer method, SQB partitions the protein-protein interaction network into a mount of Distance-2-Subgraphs , each for one vertex, and only containing two kinds of nodes: those being connected with the vertex (we call them the direct neighbors), and those being reachable from the vertex by passing just one other node (we call them distance-2-neighbors). Next, through top-down and branch-and-bound methods, SQB tries to find the quasi-bicliques from all the Distance-2-Subgraphs. At last, SQB merges the redundant ones in the quasi-biclique clusters. We applied our algorithm on the Saccharomyces cerevisiae dataset and obtained 2754 distinct quasi-bicliques.

Figure 1. An all-versus-all predicted interaction subnetwork.
The organization of this paper is as follows. Section 2 states the maximum Quasi-biclique problem. Section 3 describes the SQB algorithm for finding the maximum quasi-bicliques. Section 4 reports the results of the application of SQB algorithm on a real proteomic data. The paper ends with conclusions and the future work.

2. MAXIMUM QUASI-BICLIQUE PROBLEM

We use a simple graph like [14] to describe a protein-protein interaction network. A vertex represents a kind of protein and an edge means there is an interaction between two kinds of proteins. Quasi-biclique is a graph $G=(V, E)$, in which $V$ can be divided into two non-empty sets $\{V_1, U_1\}$ and every vertex in $V_1$ directly links to nearly every vertex in $U_1$. The question of finding maximum quasi-biclique in a graph $G=(V, E)$ can be formalized as following function.

$$\max f(G(V, E))$$

$$f(G(V, E)) = nm,$$

subject to

$$n = |V_1|, m = |U_1|, n + m \leq |V|,$$

$$V_1 \cap U_1 = \emptyset, V_1 \subset V, U_1 \subset V$$

where $|V|$ denotes cardinality of the vertex set of the input graph, $n$ and $m$ should be greater than 1 and lower than $|V|/2$. A quasi-biclique is measured by the value of $nm$ which actually is the number of interacting edges between two sets. In the following, we denote a quasi-biclue as QB ($V_1, U_1$).

3. SQB ALGORITHM

The main method of SQB is Divide-and-Conquer, which includes three parts. The first one is to seek every vertex’s Distance-2-Subgraph from a graph. The second one is to find every vertex’s quasi-biclique from its Distance-2-Subgraph. The third one is to merge solutions; after finding every vertex’s quasi-bicliques, SQB puts them together, removes the similar ones, and obtains the quasi-bicliques of the whole graph. The three parts of SQB are detailed in the following.

3.1. Finding Distance-2-Subgraph

As some graph, especially the biological protein-protein interaction network, is very large, the process on the graph will need a very large memory space so it is not feasible in common applications. But it is obvious that the distance between any two vertexes in a quasi-biclique is not greater than 2. So if we want to find a quasi-biclique which includes a specific vertex, we only need to consider the vertex and its related neighbors. The related neighbors are vertexes which are less than 3 in distance to the specific vertex. The induced subgraph, which consists of the vertex and its related neighbors, is denoted as Distance-2-Subgraph. The edge status between any two vertexes in an induced subgraph is the same as that in the original graph. SQB needs to find every vertex’s Distance-2-Subgraph in order to obtain its maximum quasi-biclique.

3.2. Detecting Maximum Quasi-bicliques

After finding every vertex’s Distance-2-Subgraph, SQB begins to find the quasi-biclique. This process, detecting quasi-bicliques, is the essential part of SQB. SQB uses the size $(nm)$ to measure a quasi-biclique and it is crucial to know the specific value of $n$ and $m$ of a maximum quasi-biclique. As $n$ and $m$ are in a limited range, SQB tests the values of $n$ and $m$ from the upper limit to the lower one. If a graph has a quasi-biclique $QB(|V|=n, |U|=m)$, the vertexes in the graph with degree lower than $n$ and $m$ should not be in the QB, so SQB removes these smaller vertexes during the process. Furthermore, if the test value of $n$ and $m$ are greater, SQB can remove more vertexes and increase the speed of the process.

Before explaining our program, we introduce how to split a graph. We use a complex data structure CD to store the V1 set, U1 set and the induced subgraph G of V1 set and U1 set. The program splits the graph at the V1 set, and U1 set in turn. The program chooses the vertex in V1 or U1 with largest degree and labels it so that next time, the program avoids splitting at the same vertex again. For example, if the program chooses v15 as the candidate vertex, it then produces four sets V150, V151, U150, and U151. The first set V150 includes v15 and vertexes in V1 which has a distance of 2 to vertex v15. The second set V151 consists of elements in V1 except v15. The third set U150 contains vertexes in U1 which is the direct neighbor of vertex v15. The fourth set U151 is the same as U1. Next, the program produces induced graph G150 which contains vertexes V150 and U150, then puts V150, U150, G150 into data structure CD150(V150, U150, G150). In the same way, it gets another data structure CD151(V151, U151, G151).

The algorithm of detecting quasi-bicliques is listed in the end of this subsection. The algorithm consists of a FOR loop that begins from 20 to 2. (20 is an experimental value which should be increased with the growing of nodes of the input graph). At first SQB uses the sub-function Search_k_Quasi_Bicliques(G, k) to test whether the graph contains quasi-bicliques in which the $|V|\geq20$ and $|U|\geq20$. If the sub-function finds it’s true, the FOR loop terminates, otherwise the FOR loop decreases the test value by one and continues to test, until the sub-function finds quasi-bicliques or the test value lower than 2.

The sub-function func Search_k_Quasi_Bicliques(G, k) is the key component of SQB. At first, the input graph G’s vertex set is divided into two parts, V1 and U1. Next, V1 and U1, and G are put into complex data structure CD(V1, U1, G). CD is put into a buffer BUFFER. Next, the program go into a WHILE loop. This loop’s terminate condition is that the buffer is empty. During the loop, first, the program removes one element from BUFFER and puts it into CD0(0, 0, G0), then deletes vertex in V01 with
degree lower than \( k \) and deletes vertex in \( U01 \) with degree lower than \( k \). Next, the program judges whether \( CD0(V01, U01, G0) \) is a quasi-biclique. If the \( CD0 \) is a quasi-biclique and its size greater than current maximum value, the program outputs \( CD0 \) and puts the current maximum value as \( CD0 \)'s size. If it is not a quasi-biclique and its size greater than current maximum value, the program splits the \( CD0 \) into two units (\( CD01, CD02 \)) and puts them into the BUFFER. Otherwise, the program discards the left smaller ones.

**Part of SQB Algorithm**

**Input.** Graph \( G(V, E) \)

**Output.** The maximum quasi-bicliques in \( G(V, E) \)

```java
func Search_Maximum_Quasi_Bicliques() =
    for tt from 20 to 2 step-1 do
        OUT = Search_k_Quasi_Bicliques(G, tt)
        If OUT is not empty
            Output OUT and Stop the program
        end for
    end func Search_Maximum_Quasi_Bicliques()

func Search_k_Quasi_Bicliques(G, k) =
    Divide vertex set \( V \) into two sets, \( V1 \) and \( U1 \)
    Put \( V1, V2, G \) into Complex Data Structure \( CD(V1, U1, G) \)
    Put \( CD \) into Buffer BUFFER
    While (BUFFER is not empty)
        Remove first element in BUFFER to \( CD0(V01, U01, G0) \)
        Delete vertex in \( V01 \) with degree lower than \( k \)
        Delete vertex in \( U01 \) with degree lower than \( k \)
        If \( CD0(V01, U01, G0) \) is Quasi_Bicliques
            then output \( CD0 \)
        Else if \( CD0(V01, U01, G0) \) is not empty
            then Split \( CD0 \) at one unlabeled vertex
            put \( CD01, CD02 \) into BUFFER
        end if
    end while
    end func Search_k_Quasi_Bicliques()
```

3.3. **Pruning Redundant Quasi-bicliques**

Through the above steps, SQB obtains every vertex’s quasi-bicliques. As some vertexes might have similar quasi-bicliques, SQB needs to remove redundant ones and obtains the overall distinct quasi-bicliques of the whole graph. SQB deletes one between any two quasi-bicliques \( QB1(V1, U1) \) and \( QB2(V2, U2) \) if they meet the following rules:

- If \( \{(V1 \subseteq V2) \land (U1 \subseteq U2)\} \lor \{(V1 \subseteq U2) \land (U1 \subseteq V2)\} \) is true, then delete \( QB1 \).
- If \( \{(V2 \subseteq V1) \land (U2 \subseteq U1)\} \lor \{(V2 \subseteq U1) \land (U2 \subseteq V1)\} \) is true, then delete \( QB2 \).

The first rule means that if \( V1 \) is \( V2 \)'s subset and \( U1 \) is \( U2 \)'s subset, or if \( V1 \) is \( U2 \)'s subset and \( U1 \) is \( V2 \)'s subset, then \( QB1 \) is a part of \( QB2 \), \( QB1 \) can be deleted. The second rule is opposite to the first one. Otherwise, if two quasi-bicliques match neither of the above two rules, SQB keeps both of them.

After the pruning operation, SQB obtains the distinct quasi-bicliques of the whole graph and the biggest one is the optimum one of the whole graph.

4. APPLICATION OF SQB

The experiment was done on our web server which consisted of two Pentium 2 PCs with 4.8 GHZ CPU and 2G RAM. The Saccharomyces cerevisiae dataset Y78, derived from [15], consists of 78,390 protein-protein interactions, including 5321 proteins. During our experiments, we removed vertexes with degree 1 because they could not produce a biclique. The input graph of our program is with node number 4546. At first, we produced 4546 distinct Distance-2-Subgraphs according to every vertex’s neighbors and their neighbors. The maximum subgraph is with 3164 nodes and the average value is 746.1. So the questions are very tough.

About eighty percent of the vertexes have a process time less than 20 seconds and half of them are processed within 1 second. The average time is about 13.4 seconds. Giving the large input graphs, the performance is very remarkable.

During our experiments we predicted 5616 quasi-bicliques which include empty or redundant ones. A small number of vertexes have more than one maximum quasi-bicliques, so the number of quasi-bicliques is greater than 4546, the number of Distance-2-Subgraphs.

| \(|V|\)=26 | YBL038W YBR251W YBR283C YDL136W YDL191W YEL050C YGL103W YGL123W YGR034W YGR220C YIL018W YIL021W YJL063C YLR075W YLR344W YLR378C YMR260C YNL178W YNL284C YNR037C YOL040C YOL127W YOR063W YPL131W YPL183W-A YPR110C |
| \(|U|\)=62 | YBL087C YBL091C YBR031W YBR048W YBR146W YCR031C YDL061C YDL083C YDL140C YDL202W YDR012W YDR025W YDR101C YDR116C YDR226W YDR237W YDR418W YDR450W YEL054C YER117W YER170W YFL001W YGL063W YGL068W YGL135W YGL147C YGR085C YHR147C YIL133C YJL177W YJL190C YJL191W YKL009W YKL024C YKL170W YKL180W YLR244C YLR340W YLR367W YLR388W YML010W YML025C YML026C YMR143W YMR158W YMR188C YNL067W YNL069C YNL081C YNL177C YNL185C YNL306W YOR116C YOR150W YOR151C YOR207C YOR341W YPL212C YPL220W YPR010C YPR102C YPR166C |
The size of quasi-biclique is measured by the product of \( n=|V| \) and \( m=|U| \). Figure 2 shows the size of every vertex’s maximum quasi-biclique. The average value is 115.8. The largest one is with size 1612=26*62 which includes protein YPL212C and is listed in Table 1. Every vertex in the protein set V touches every one in the set U. During our experiments, we thought the quasi-biclique with size lower than 4*4 is easy to occur by random and should be discarded. At last we obtained 2754 distinct maximum quasi-bicliques which are available on our website http://biod.whu.edu.cn/pub/QuasiBiclique.txt.

5. CONCLUSION

In this study, we developed Searching Quasi-Bicliques (SQB) algorithm to detect maximum quasi-biclique from a large scale protein-protein interaction network. SQB uses the Divide-and-Conquer method to process data. Combination of Top-down method and the branch-and-bound method greatly reduces the search space. We successfully applied it to the analysis of Yeast proteomic data and obtained many quasi-bicliques, which might provide meaningful clues to potential biological users.

Although we obtained some quasi-bicliques, they might not be global optimum ones because the results are influenced by the choice of start splitting vertex. In addition, we might integrate more biological features, such as the proteins’ functional category, to analyze the mechanism of the quasi-biclique in the cell.

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Using schema transformation pathways for biological data integration

Hao Fan & Fei Wang

ABSTRACT

In web environments, proteomics data integration in the life sciences needs to handle the problem of data conflicts arising from the heterogeneity of data resources and from incompatibilities between the inputs and outputs of services used in the analysis of the resources. The integration of complex, fast changing biological data repositories can be potentially supported by Grid computing to enable distributed data analysis. This paper presents an approach addressing the data conflict problems of proteomics data integration. We describe a proposed proteomics data integration architecture, in which a heterogeneous data integration system interoperates with Web Services and query processing tools for the virtual and materialised integration of a number of proteomics resources, either locally or remotely. Finally, we discuss how the architecture can be further used for supporting data maintenance and analysis activities.

Keywords: Evoked potentials (EPs), Alpha stable distribution, Blind source separation, Minimum dispersion (MD), Fractional lower order statistics (FLOS)

1. INTRODUCTION

In the life sciences, along with the deepening investigation into human genome, especially into their functionalities, study on proteomics becomes an important key issue of life sciences research. Since protein is the reflector of genome functionalities, research on proteomics is to investigating the structures and functions of protein, in order to interpret the variation mechanism of a life in physiologies or pathologies. Issues relating to protein innate existence forms and activity patterns, such as interpreting gene modifiers, protein interactions and configurations, require the solution of study on the protein complement of the genome, i.e. proteomics, which is also an essential component of any comprehensive functional genomics study targeted at the elucidation of biological functions.

Proteome databases and genome pools are generally used for proteomics research. However, global protein expression analysis refers to any experiment in which the expression of all genes is monitored simultaneously, which generate large amounts of data, but there is no universal system for the description of gene expression profiles. Global protein expression data are obtained predominantly as signal intensities on 2D protein gels.

A large amount of biological information is available over the Internet, but the data are widely distributed and it is therefore necessary to have efficient mechanisms for data integration and data retrieval. Grid computing technologies are becoming established which enable distributed computational and data resources to be accessed in a service based environment. These technologies offer the possibility of analysis of complex distributed post-genomic resources. To support transparent access, however, such heterogeneous resources need to be integrated rather than simply accessed in a distributed fashion.

This paper introduces a proteomics data integration architecture, using a heterogeneous data integration system interoperates with Web Services and query processing tools for the virtual and materialised integration of a number of local and remote proteomics resources. We also discuss how the architecture can be further used for supporting data maintenance and analysis activities, i.e. processing user queries, tracing data linages, and maintaining data incrementally.

Paper outline: Section II gives an overview of protein database resources available in the Internet. Section III presents a web-based heterogeneous data integration architecture, including the BAV data integration technologies and Web services, the transformation pathways for creating the global schema, and the key issues addressed in the architecture for applying to biological data integrations. Finally, Section IV gives our conclusions and directions of future work.

2. OVERVIEW OF PROTEOME DATABASE RESOURCES

Proteome databases receive more and more attentions in proteomics research, which strives to provide a high level of annotation, such as the description of the function of a protein, its domains structure, post-translational modifications variants, etc., a minimal level of redundancy and high level of integration with other databases [2].
2.1. Protein Sequence Databases

2.1.1. General sequence databases

EXProt (see http://www.cmbi.kun.nl/EXProt/) is aiming at including only proteins with an experimentally verified function, which is a non-redundant protein database containing a selection of entries from genome annotation projects and public databases. Its each entry has a unique ID number and contains information about the species, amino acid sequence, functional annotation.

UniProt (see http://www.uniprot.org) is formed by unifying activities of the Swiss-Prot1, TrEMBL2, and PIR3 protein databases, which provides a central resource on protein sequences and functional annotation with three database components, each addressing a key need in protein bioinformatics.

TCDB (see http://www.tcdb.org) is a curated relational database containing sequence, classification, structural, functional and evolutionary information about transport systems from a variety of living organisms. TCDB is a repository for information compiled from more than 10; 000 references, encompassing approximately 3; 000 representative transporters and putative transporters, classified into over 400 families.

2.1.2 Protein properties

iProLINK (see http://pir.georgetown.edu/iprolink) facilitates text mining research in the area of literature-based database curation, named entity recognition, and protein ontology development. This collection of annotated data sources can be utilized by computational and biological researchers to explore literature information on proteins and their features.

PFD (see http://www.foldomics.org/pfd/) has a database structure allows visualization of folding data in a useful and novel way, with aims of facilitating data mining and bioinformatics approaches, which is a searchable collection of all annotated structural, methodological, kinetic and thermodynamic data relating to experimental protein folding studies.

PINT (see http://www.bioinfodatabase.com/pint/) contains data of several thermodynamic parameters along with sequence and structural information, experimental conditions and literature information. Each entry contains numerical data for the free energy change, dissociation constant, association constant, enthalpy change, heat capacity change and so on of the interacting proteins upon binding, which are important for understanding the mechanism of protein-protein interactions.

2.1.3 Protein sequence motifs

PROSITE (see http://www.expasy.org/prosite) is a large collection of biologically meaningful signatures described as patterns or profiles. Each signature is linked to a documentation that gives useful biological information on the protein family, domain, or functional site identified by the signature.

Blocks (see http://blocks.fhcrc.org) are ungapped multiple alignments corresponding to the most conserved regions of proteins, which consists of blocks constructed from documented families of related proteins. A blocks multiple alignment consists of unaligned conserved regions separated by unaligned regions of variable size.

PRINTS (see http://www.bioinf.man.ac.uk/dbbrowser/) houses a collection of protein family fingerprints, which may be used to make familial and tentative functional assignments for uncharacterised sequences. It specializes in the provisional of hierarchical classifications of protein superfamilies, allowing fine-grained diagnoses, and provides the bulk of the hierarchical family annotation in InterPro. PRINTS also underpins the Blocks database from Seattle and eMOTIF resource from Stanford.

2.1.4. protein domains and classifications:

iProClass (see http://pir.georgetown.edu/iproclass/) provides an integrated view of protein information and serves as a bioinformatics framework for data integration and associative analysis of proteins. It presents value-added descriptions of all proteins in UniProtKB and contains comprehensive, up-to-date protein information derived from over 90 biological databases. The source databases include those of protein sequence, family, function, pathway, protein-protein interaction, complex, post-translational modification, protein expression, structure and structural classification, gene and genome, gene expression, disease, ontology, literature, and taxonomy.

2.2. Protein Structure Databases

PDB (see http://www.rcsb.org/pdb/) is the single worldwide archive of structural data of biological macromolecules. A description of the architecture and functionality of the systems used to collect, archive, distribute, and query the data were described previously [3].

SCOP (see http://scop.mrc-lmb.cam.ac.uk/scop) provides a comprehensive and detailed description of the evolutionary and structural relationships of the proteins of known structure. It embodies an evolutionary classification produced by human experts. This allows users to use a theory of protein evolution that encompasses our knowledge of the great variety, and the full extent, of the different types of changes that take place during evolution.

CATH (see http://www.biochem.ucl.ac.uk/bsm/cath new) currently contains 34; 287 domain structures classified into 1; 383 superfamilies and 3; 285 sequence families. Each structural family is expanded with domain sequence relatives recruited from GenBank using a variety of efficient sequence search protocols and reliable thresholds. New sequence search protocols have been designed, based on these intermediate sequence libraries, to allow more regular updating of the classification.

2.3. Proteomics Resources

PEDRo (see http://pedrodb.man.ac.uk:8080/pedrodb) provides access to a collection of descriptions of experimental data sets in proteomics [8]. It was one of the first databases used for storing proteomics experimental data and has also been used as a format for exchanging
The integration of such a comprehensive data model, in addition to its data content, provides means for capturing a significant proportion of the proteomics information that is captured by other proteomics repositories.

**gpmDB** (see http://gpmdb.thegpm.org) is a publicly available database with over 2,200,000 proteins and almost 470,000 unique peptide identifications [4]. Although the gpmDB is restricted to minimal information relating to the protein/peptide identification, it provides access to a wealth of interesting and useful peptide identifications from a range of different laboratories and instruments.

**PepSeeker** (see http://nwsr.smith.man.ac.uk/pepseeker) is a database targeted directly at the identification stage of the proteomics pipeline. It captures the identification allied to the peptide sequence data, coupled to the underlying ion series and as a result it is a comprehensive resource of peptide/protein identifications [11]. The repository currently holds over 50,000 proteins and 50,000 unique peptide identifications.

**PRIDE** (see http://www.ebi.ac.uk/pride/) is a database of protein and peptide identifications that have been described in the scientific literature. These identifications will typically be from specific species, tissues and sub-cellular locations, perhaps under specific disease conditions and may be annotated with supporting mass spectra. PRIDE can be searched by experiment accession number, protein accession number, literature reference and sample parameters including species, tissue, sub-cellular location and disease state. Data can be retrieved as machine-readable PRIDE or mzData XML, or as human-readable HTML.

### 3. DATA INTEGRATION ARCHITECTURE

**Figure 1** illustrates a heterogeneous data integration architecture in Web environments. In this architecture, remote data sources use web service platforms producing data exchange and access processes with external users. The BAV data integration system interoperates with web services enabling query processing tools for the virtual and materialised integration of a number of distributed data resources.

Specially, in the scenario of integrating local databases, BAV wrappers apply to the local data sources directly, so that extracting data and data structure from data sources and producing global user queries. On the other hand, in the scenario of web data integrations, BAV wrappers exchange data with web services so that access the remote data sources. Both virtual and materialised integrated views can be created by the data integration system.

In this section, we discuss how this architecture can be proposed used for integrating proteomics data resources.

### 3.1. Data Integration Technologies

**BAV Data Integration.** Up to now, most data integration approaches have been either **global-as-view (GAV)** or **local-as-view (LAV)**. In GAV, the constructs of a global schema are described as views over local schemas. In LAV, the constructs of a local schema are defined as views over a global schema. One disadvantage of GAV and LAV is that they do not readily support the evolution of both local and global schemas. In particular, GAV does not readily support the evolution of local schemas while LAV does not readily support the evolution of global schemas. Furthermore, both GAV and LAV assume one common data model for the data transformation and integration process, typically the relational data model.

**Both-as-view (BAV)** is a new data integration approach based on the use of reversible sequences of primitive schema transformations [10]. From these sequences, it is possible to derive a definition of a global schema as a view over the local schemas, and it is also possible to derive a definition of a local schema as a view over a global schema. BAV can therefore capture all the semantic information that is present in LAV and GAV derivation rules. A key advantage of BAV is that it readily supports the evolution of both local and global schemas, allowing transformation sequences and schemas to be incrementally modified as opposed to having to be regenerated.

Another advantage is that BAV can offers the capability to handle virtual, materialised and indeed hybrid data integration across multiple data models. This is because BAV supports a low-level *hypergraph-based data model (HDM)* and provides facilities for specifying higher-level modelling languages in terms of this HDM. For any modelling language $M$ specified in this way, the approach provides a set of primitive schema transformations that can be applied to schema constructs expressed in $M$. In particular, for every construct of $M$ there is an add and a delete primitive transformation which add to/delete from a schema an instance of that construct. For those constructs of $M$ which have textual names, there is also a rename primitive transformation. BAV schemas can be incrementally transformed by applying to them a sequence of primitive transformations, each adding, deleting or renaming just one schema construct.
Each add and delete transformation is accompanied by a query specifying the extent of the added or deleted construct in terms of the rest of the constructs in the schema. This query is expressed in a functional query language, IQL, which is a comprehensions-based language and we refer the reader to the reference [9] for details of its syntax, semantics and implementation. Such languages subsume query languages such as SQL-92 and OQL in expressiveness [1].

Also available are extend and contract primitive transformations which behave in the same way as add and delete except that they state that the extent of the new/removed construct cannot be precisely derived from the other constructs present in the schema. More specifically, each extend and contract transformation takes a pair of queries that specify a lower and an upper bound on the extent of the construct. The lower bound may be Void and the upper bound may be Any, which respectively indicate no known information about the lower or upper bound of the extent of the new construct. The queries supplied with primitive transformations can be used to translate queries or data along a transformation pathway.

A sequence of primitive transformations from one schema $S_i$ to another schema $S_j$ is termed a pathway from $S_i$ to $S_j$. All source, intermediate, and integrated schemas, and the pathways between them, are stored in a Schemas & Transformations Repository.

**Web Services.** Web services provide a standard means of interoperating between different software applications (see http://www.w3.org/TR/ws-arch/), running on a variety of platforms and/or frameworks. A Web service is a software system designed to support interoperable machine-to-machine interaction over a network. It has an interface described in a machine-processable format (specifically Web Services Description Language, WSDL). Other systems interact with the Web service in a manner prescribed by its description using SOAP messages, typically conveyed using HTTP with an XML serialization in conjunction with other Web-related standards.

### 3.2. Data Integration Using Transformation Pathways

The aim of the proteomics data integration is to build technologies providing an environment for integrating proteomics data, constructing and executing analyses over such data, and a library of proteomics-aware components that can act as building blocks for such analyses. A web-based architecture is enabling existing proteomics data resources, creating new resources, producing middleware technologies for the integration of protein data resources — including tools for data integration, data analysis, producing user queries, visualisation applications and other types of client for biologist end users.

This section gives examples of using transformation pathways for creating a global schema. Supposing the table ProteinHit (all peptides matched, expect, score, threshold, protein, peptideId), indicated as $hh$proteinhitii, in the global schema, which is composed of data originally from four different databases, PEDRo, gpmDB, PepSeeker and Pride. The following transformation pathways are used to create the $hh$proteinhitii schema, in which $id2lsid$ is an IQL build-in function used to transform id features in source schemas into lsid feature in the global schema.

1. **Creating ProteinHit from PEDRo database:**

   ```
   add (uproteinhit)
   [id2lsid k] k \rightarrow (proteinhit)
   add (uproteinhit, lsid)
   [k, k] k \rightarrow (proteinhit)
   add (uproteinhit, all_peptides_matched)
   [id2lsid k, x] k \rightarrow (proteinhit, all_peptides_matched)
   ext (uproteinhit, expect) Void
   add (uproteinhit, score)
   [id2lsid k, x] k \rightarrow (proteinhit, score)
   ext (uproteinhit, threshold) Void
   add (uproteinhit, protein)
   [id2lsid k, x] k \rightarrow (proteinhit, protein)
   add (uproteinhit, peptideId)
   [id2lsid k, x] k \rightarrow (proteinhit, peptideId)
   ```

2. **Creating ProteinHit from gpmDB database:**

   ```
   add (uproteinhit)
   [id2lsid k] k \rightarrow (protein)
   add (uproteinhit, lsid)
   [k, k] k \rightarrow (proteinhit)
   ext (uproteinhit, all_peptides_matched) Void
   add (uproteinhit, expect)
   [id2lsid k, x] k \rightarrow (protein, expect)
   ext (uproteinhit, score) Void
   ext (uproteinhit, threshold) Void
   add (uproteinhit, protein)
   [id2lsid k, x] k \rightarrow (protein, proteinId)
   ext (uproteinhit, peptideId) Void
   ```

3. **Creating ProteinHit from PepSeeker database:**

   ```
   add (uproteinhit)
   [id2lsid k] k \rightarrow (protein)
   add (uproteinhit, idk)
   [k, k] k \rightarrow (proteinhit)
   ext (uproteinhit, all_peptides_matched) Void
   ext (uproteinhit, expect) Void
   add (uproteinhit, score)
   map \lambda (k, x) \rightarrow (id2lsid k, x) (distinct \{x1, x2\})
   \{k1, x1\} \rightarrow (proteinhit, proteinId)
   \{k2, x2\} \rightarrow (proteinhit, Score); x1 = k2)
   ext (uproteinhit, threshold) Void
   add (uproteinhit, peptideId)
   [id2lsid k, x] k \rightarrow (proteinhit, peptideId)
   ```

4. **Creating ProteinHit from Pride database:**

   ```
   add (uproteinhit)
   [id2lsid k] k \rightarrow (pride_identification)
   add (uproteinhit, lsid)
   [k, k] k \rightarrow (proteinhit)
   ext (uproteinhit, all_peptides_matched) Void
   ext (uproteinhit, expect) Void
   add (uproteinhit, score)
   [id2lsid k, x] k \rightarrow (pride_identification, Score)
   add (uproteinhit, threshold)
   add (uproteinhit, threshold)
   ```
3.2. Using Schema Transformation Pathways

In the previous section we showed how schema transformation pathways can be used for expressing the process generating a global schema. In this section, we discuss how the transformation pathways can be used for the following further activities.

1) Schema Evolution: A recurring issue within the data integration architecture is that the source schemas will change as the owners of these autonomous data sources evolve them over time. Changes in global schemas may also be needed in order to support new requirements of the client components. An advantage of the BAV approach over GAV or LAV data integration is that it readily supports the evolution of both source and global schemas by allowing transformation pathways to be extended — this means that the entire integration process does not have to be repeated, and the schemas and pathways can instead be ‘repaired’. This process can be handled largely automatically, except in cases where new information content is being added to schemas where domain or expert human knowledge is needed regarding the semantics of new schema constructs.

2) Transforming Data Schemas: In previous work [7], we show that transformation pathways can be used for expressing the process generating global schemas in biological data integration environments. Each transformation step is accompanied by an add/delete operation with a query specifying the extent of the added or deleted construct in terms of the rest of the constructs in the original schema.

3) Processing User Queries: An WS wrapper imports schema information from any data source, via web services, into a metadata repository. Thereafter, schema transformation/integration functionality can be used to create one or more virtual global schemas, together with the transformation pathways between these and the BAV representations of the data source schemas. Queries posed on a virtual global schema can be submitted to a query processor, and this interacts with web services via WS wrappers to evaluate these queries. After the integration of the data sources, the user is able to submit to the query processor a query to be evaluated with respect to the virtual global schema.

4) Tracing Data Lineage: In the data integration architecture, proteomics data is integrated from distributed, autonomous and heterogeneous data sources, in order to enable analysis and mining of the integrated information. However, in addition to analyzing the data in the integrated Grid, we sometimes also need to investigate how certain integrated data was derived from the data sources, which is the problem of data lineage tracing (DLT).

In [5], we present a DLT approach which is to use the individual steps of these pathways to compute the lineage data of the tracing data by traversing the pathways in reverse order one step at a time. In particular, suppose a data source LD with schema LS is transformed into a global database GD with schema GS, and the transformation pathway $LS \rightarrow GS$ is $ts_1, \ldots, ts_n$. Given tracing data $td$ belonging to the extent of some schema construct in GD, we firstly find the transformation step $ts$ which creates that construct and obtain $tds$ lineage, $dl$, from $ts$. We then continue by tracing the lineage of $dl$ from the remaining transformation pathway $ts_i, \ldots, ts_n$. We continue in this fashion, until we obtain the final lineage data from the data source LD.

5) Maintaining Integrated Data Incrementally: The global schema might be materialised by creating an integrated database. A problem relating to materialised integrated data is view maintenance. The materialised integrated data need to be maintained either when the data of a data source changes, or if there is an evolution of a data source schema. If the source data is updated, the integrated data has to be refreshed also so as to keep it up-to-date.

If a materialised construct $c$ is defined by an query $q$ over other materialised constructs, [6] gives formulae for incrementally maintaining $c$ if one its ancestor constructs $ca$ has new data inserted into it (an increment) or data deleted from it (a decrement). We actually do not use the whole view definition $q$ generated for $c$, but instead track the changes from $ca$ through each step of the pathway. In particular, at each add or rename step we use the set of increments and decrements computed so far to compute the increment and decrement for the schema constructed being generated by this step of the pathway.

6) Extendable Exploitations: In peer-to-peer (P2P) systems, a number of autonomous servers, or peers, share their computing resources, data and services. The loose and dynamic association between peers has meant that, to date, P2P systems have been based on the exchange of files identified by a limited set of attributes. The lack of information about the data within these files makes it impossible to support general-purpose mechanisms by which peers can exchange and translate heterogeneous data. How the heterogenous data integration architecture can be used to integrate proteome data in different peers would be the extendable exploitation of our work.

4. CONCLUSION

In this paper, we give a brief overview of protein database resources available in the Internet, and present an architecture combining web services and a data integration software tools over the autonomous data resources which enables distributed query processing together with the resolution of semantic heterogeneity over autonomous data resources. We also discuss the key issues of applying the architecture to biological data integrations, namely schema evolution, transforming data schemas, processing user queries, tracing data lineage, maintaining integrated data incrementally, and extendable exploitations.

The final platform will provide researchers with more information than any of the resources alone, so allowing them to perform analyses that were previously prohibitively difficult or impossible. This integration process
both builds on and provides impetus to the development of data standards in the proteomics and related domains.

Future work incudes designing and implementing wrappers for extracting data and data structure over web services, implementing DLT and IVM algorithms over the architecture, and investigating into extending the system into P2P environments.

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