Report of the 2nd RCM on "Nanoscale radiation engineering of advanced materials for potential biomedical applications"

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Foreword

There are critical needs for advanced materials in the area of biomaterial engineering, primarily in generating biomaterials of enhanced specific functionalities, improved biocompatibility, and minimal natural rejection but with enhanced interfacial adhesion. These can be achieved by introduction of proper functionalities at the nanoscale dimensions for which, due to their characteristics, radiation techniques are uniquely suited. Accordingly, many of the IAEA Member States (MS) have interest in creating advanced materials for various health-care applications using a wide array of radiation sources and their broad expertise.

In seeking new knowledge to advance the field and tackle this specific problem, to collaborate to enhance the quality of the scientific research and improve their efficiency and effectiveness, MS had requested the support of the IAEA for such collaboration. Based on these requests, and the conclusions and recommendations of the Consultant’s meeting on “Advanced Materials on the Nano-scale Synthesized by Radiation-Induced Processes”, held on 10-14 December 2007, the present CRP was formulated and started in 2009. The first RCM was held in 30 March – 03 April 2009, in Vienna, where the work plan for both individual participants and collaborations were discussed and accepted, as reported in the Meeting Report published as IAEA Working Material (http://www-naweb.iaea.org/napc/iachem/working_materials.html).

The second RCM was held on 15-19 November 2010, Paris, France, and was attended by 17 participants (chief scientific investigators or team members) and one cost-free observer from Brazil. The participants presented their research achievements since the first RCM, centred on the main expected outputs of this CRP: a. Methodologies to prepare and characterize nanogels; nanoparticles and nanoporous membranes, as well as to synthesize and modify nanoparticle surfaces by attaching organic ligands by radiation; b. Methodologies to radiation synthesize polymeric, inorganic and hybrid nanocarriers, providing a controlled loading and improved releasing rate of drugs; and c. Demonstration of novel functional surfaces for cell-sheet engineering fabricated by utilizing advanced radiation technology, towards improved cell-matrix interactions and cell function control. This meeting report presents in its first part the summaries of the achievements, the conclusions reached and recommendations given, the various collaborations realized among the participants, as well as the list of scientific publications. The second part of the report consists of the full reports of the participants work during the past year. The participant from Egypt was unable to attend this meeting, but made his contribution available for inclusion in this report.

The IAEA wishes to thank all participants of the Meeting for their valuable contributions. Special thanks are due to the host, who made this event a memorable success. The IAEA officer responsible for this Research Coordination Meeting was Agnes Safrany of the Division of Physical and Chemical Sciences.
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EXECUTIVE SUMMARY

1. BACKGROUND

Nanoparticles and nanostructures are not entirely new, while the ability of humans to work, measure and manipulate at the nano-scale is new. Radiation technologies, already established in materials processing, have properties uniquely suited for the creation and characterization of new functional materials on the nanoscale.

For example, low energy ion beam enables the fabrication of 3D structures, while high energy ion beam is used for preparation of ion-track membranes and nanowires. Membranes containing a single pore, or any number of pores up to 109 pores/cm² of highly uniform pore size are already commercially available. By further modification there are endless possibilities for creating track membranes with special properties and functions. These membranes may also be used as template materials for the synthesis of micro- and nanostructures, in the form of wires or tubules. Magnetic, conducting and superconducting nanowires and nanotubules, single or in array have been manufactured this way. These processes are well developed in Japan, the USA, Germany, France and some other MSs.

Generation of nanoparticles using radiolytic techniques has been known for several decades now. Strategies to produce nanoparticles of metallic and semiconductor materials were developed and production of metallic core-shell or alloyed metallic nanoparticles is possible. Radiation technique offers unique advantages by creating a wide range of species with redox levels not achievable by other means. Moreover, by adjusting the dose and dose rate, a better control and fine-tuning of the particle size and size distribution is possible.

The use of pulsed irradiation to synthesize polymer nanogels was initiated in Poland, accepted and further developed in laboratories in Germany, Hungary, Kazakhstan, Malaysia, US and others. Properties of polymer nanogels, as compared to single macromolecules or non-crosslinked nanoparticles are: stability of shape and size, ability to react to external stimuli, ability to host small molecules and to release them in a controlled way, ability to form non-flat, structured surfaces and stability against degradation. These properties make them ideally suited in health-care applications, for example: in diagnostics as carriers of contrast agents or markers, in therapy as stimuli-responsive coatings for drug- or gene delivery, encapsulation and wound healing. These nanogels can be additionally functionalized by coupling with suitable biomolecules for targeting and imaging, can be used as additives to synovial fluids and intravenous drug carrier.

Nanoscale grafting of environmental sensitive hydrogel onto a surface of cell culture dishes (by gamma or electron irradiation), enables the harvesting of these cells by a simple change in the temperature or pH of the cell-culture media. In this way, no proteolytic enzymes are needed that often damage the cells, and cells can be harvested in a continuous sheet form. Such cell-sheets overcome the limitations of conventional tissue engineering methods such as the use of single-cell suspension injection, and have already shown good results in regenerative medical applications in Japan. Skin and corneal defects have been treated with transplantable cell sheets fabricated on these surfaces. Severe heart failure was also treated with cell sheets fabricated from patient’s own skeletal myoblasts. With further improvements of stimuli-responsive culture surfaces reconstruction of more complex tissues will be possible, leading to treatments of a wider range of
diseases. This process generated a huge interest in a number of MS, which are now exploring various routes (US, Turkey, Hungary, Poland, Italy, and others.) for preparation of surfaces suitable for cell sheet engineering and requested the guidance and coordination of their efforts from the IAEA.

The International Atomic Energy Agency’s Industrial Applications and Chemistry Section of the Division of Physical and Chemical Sciences, Department of Nuclear Sciences and Applications, has recognized the challenges and opportunities of these new scientific and technological developments, organized several Technical and Consultant’s Meetings and published a TECDOC entitled “Emerging applications of radiation in nanotechnology”.

Based on the requests and information received from the member states and the conclusions and recommendations of the Consultant’s meeting on “Advanced Materials on the Nano-scale Synthesized by Radiation-Induced Processes”, held on 10-14 December 2007, the present CRP was formulated and started in 2009. The first RCM was held in 30 March – 03 April 2009, in Vienna, where the work plan for both individual participants and collaborations were discussed and accepted, as reported in the Meeting Report published as IAEA Working Material (http://www-naweb.iaea.org/napc/iachem/meetings/RCMs/RC-1124-2_report_complete.pdf).

The second RCM was held on 15-19 November 2010, Paris, France, and was attended by 17 participants (chief scientific investigators) and one cost-free observer from Brasil. The participants presented their research achievements since the first RCM, centred on the main expected outputs of this CRP:

1. Methodologies to prepare and characterize nanogels; nanoparticles and nanoporous membranes, as well as to synthesize and modify nanoparticle surfaces by attaching organic ligands by radiation:

Six MS institutions (Brasil, China, France, India, Serbia, USA) reported achievements regarding nanoparticles, such as successful synthesis of spherical nanoparticles of silver (Ag) of controlled size and shape inside crosslinked polymer gels, and onto radiation-grafted single- and multi-walled carbon nanotubes (CNT). Organic core-shell nanoparticles were obtained from PVDF and PAA, while inorganic nanoparticles were made of Prussian blue and barium sulphate. Nanocomposite nanoparticles have been produced by radiation induced oxidative polymerisation of aniline or pyrol onto silica nanoparticles surfaces in the presence of gold or platinium and ruthenium salts.

Nanoporous membranes were successfully fabricated by ion-beam irradiation of engineering plastics (Japan), while drug-eluting polymer coatings with tailored nanostructures were radiation-polymerized and crosslinked onto medical implants (Hungary).

2. Methodologies to radiation synthesize polymeric, inorganic and hybrid nanocarriers, providing a controlled loading and improved releasing rate of drugs:

Most participants were working towards developing methodologies for nanocarrier synthesis (Argentina, France, Italy, Korea, Malaysia, Poland, Thailand, Turkey, USA), and reported synthesis of nanocarriers for drug delivery from natural polymers (albumin, chitosan, palm oil acrylates), and synthetic polymers (PEGDA, PAA, PVP, PVDF, PVA, PPy+PANI), with controlled size (10 – 300 nm) and shape (hollow spheres, hard spheres, soft gels). Improved control of the size and shape of nanocarriers was achieved by examining the mechanism of the formation of nanogel at various polymer concentrations and by using polymers of controlled molecular weight distribution, irradiated at selected conditions of pH, temperature and solubility. Additionally, introduction of multifunctional groups for improved stability, redispersibility from the dry state and
affinity for some specific drugs or biological molecules of interest for carrier applications was reported. For targeting ligands, small anti-angiogenic cyclo-peptides have been immobilized onto radio-grafted nancarriers to target especially cancer cell.

3. Demonstration of novel functional surfaces for cell-sheet engineering fabricated by utilizing advanced radiation technology, towards improved cell-matrix interactions and cell function control:

Procedures for RAFT-mediated and radiation induced grafting of stimuli-responsive polymers such as polyNIPAM and PAA onto cellulose has been established providing a close control of graft chain length and molecular weight distribution (Turkey, USA). Moreover, the introduction of graft domains on the nano-scale in fluorinated polymer substrates that could work as ion-channels was achieved.

In additional relevant developments, preparation and characterization of various nanocomposites were reported, in form of particles, membranes and films (Iran, Korea, Serbia, Thailand, Japan), by using ion beam, electron beam and gamma irradiations, for potential biosensing, biocatalytic, antibacterial, implant and wound dressing applications.

Some participants of this CRP effectively utilized relevant National and Regional TC projects to fund scientific visits, fellowships and expert missions to help their joint efforts to achieve the objective of this CRP. The participants reported the results of their research efforts in scientific conferences and symposia, and published them in scientific journals listed at the end of this section.

2. CRP OVERALL OBJECTIVE

The overall objective of this project is to support MS on the use of radiation in the synthesis, modification, and characterization of advanced materials by nanoscale control of their properties. This is in line with the objective of the project 2.5.2.2., to enhance Member State capabilities in applying radiation technology for advanced materials development and processing natural polymers into value added products.

2.1. Specific Research Objectives

The purpose of this project is to coordinate the research in MS on the use of radiation in the synthesis, modification, and characterization of advanced materials by nanoscale control of their properties, specifically:

1. to develop radiolytic methodologies for synthesis of nanoparticles and nanoporous membranes, as well as to synthesize and modify nanoparticle surfaces by attaching organic ligands,

2. to radiation synthesize polymeric, inorganic and hybrid nanocarriers, providing for controlled loading and improved releasing rate of drugs,

3. to fabricate new stimuli-responsive surfaces by radiation induced grafting on the nanoscale for cell-sheet engineering with improved cell-matrix interactions and cell-function control.

The participant groups will address and contribute to one or more of the above topics.

2.2. Expected Research Outputs

1. Methodologies to prepare and characterize nanogels; nanoparticles and nanoporous membranes, as well as to synthesize and modify nanoparticle surfaces by attaching organic ligands by radiation.
2. Methodologies to radiation synthesize polymeric, inorganic and hybrid nanocarriers, providing a controlled loading and improved releasing rate of drugs.

3. Demonstration of novel functional surfaces for cell-sheet engineering fabricated by utilizing advanced radiation technology, towards improved cell-matrix interactions and cell function control.

4. Publication of the results under the IAEA Radiation Technology Series.

3. SUMMARY OF PARTICIPANTS REPORTS

3.1. Argentina

Material requirements emerging from the field of nanotechnology were extended to control structures at nanosize level. Radiation technology has the potential to deliver enough energy to perform chemical reaction at limited spatial sub-nanometric regions. In addition to dimensions, functionalization is an important feature to render versatility to develop future applications of nanoparticle and nanogel structures.

During the first phase of this RCP we have studied the experimental conditions for the preparation and characterization of nanoparticles, by stabilization of nanostructured protein complexes using radiation technology. The effect of gamma rays on proteins was studied for many years, mainly in features related to degradation; however, soluble globular proteins have not been used as raw material for material synthesis until now. In our work, nanoparticle preparation has been done by gamma irradiation of albumin solutions in presence of some additives, such as alcohols. The minimum irradiation dose to reach nanoparticles was determined as 10 kGy under non-modified atmosphere.

The obtained protein nanoparticles had in general three-dimensional structure stabilized by covalent crosslinks, as confirmed by spectroscopy. The proposed mechanism assumes dynamic protein aggregation by the co-solvent, which is further stabilized by radiation crosslinking. Taking into account the Radiation Target Theory, direct hit onto proteins should not be enough to explain the stability of the nanoparticles and an additional indirect effect should be considered. These materials, which preserve the native protein conformational structure, can be useful in the development of diagnostic biomedical platform for analysis and/or drug delivery nanocarriers.

3.2. Brazil

Papain is a proteolytic enzyme with desirable property for wound healing purposes, such as debridement properties, ability to reduce bacterial burden and to increase granulation during tissue formation. However, low stability restricts this biomolecule application to short shelf-life formulations. Also, recent concerns have been pointed out by the FDA considering the allergenic properties of such substances and now, topical products containing papain must be registered prior to their commercialization. For such purpose, the goals of this research are to enhance stability of this enzyme and reduce the cytotoxic and allergenic profile through molecular encapsulation with B-cyclodextrin and radiation exposition, which is a technique applied to promote simultaneous crosslinking and sterilization of the hydrogel – selected media for the delivery of such complex. The encapsulation is also aimed to reduce the degradation effects attributed to the radiation exposition. On this account, the radiation influence over the bioactivity of the enzyme at different radiation dosages has been evaluated on a media and a compound based
approach. Preliminary experiments were performed to characterize the molecular encapsulation changes in the bioactivity profile of the enzyme. The establishment of a methodology to determine the enzymatic activity was one of the expected outputs and was performed by validation studies where accuracy, detection limit, precision, linearity and precision values were determined. Also, our next goal is to develop nanostructured hydrogels for the delivery of the prepared papain/cyclodextrin complex. On this account several hydrogels formulations based on different concentrations of PVP, PEG and AGAR were assayed for their swelling, gel fraction and mechanical properties. SEM analyses were performed as a complementary technique to provide more data about the structure of the developed material. Activities related to radiation synthesis of nanostructured nanoparticles were performed such as the development of biodegradable films for food and possible biomedical applications based on starch, ecoflex, PCL and PVA polymers. Another activity for the latter application was the development of high melt strength PP with intercalated nanoclay. The next step of this research involves the adequate hydrogel nanostructuring suitable for papain/cyclodextrin complex delivery to be characterized by Tg, Modulus and using the Flory-Rehner Equation, as well as determine the adequate molar ratio for the complex formation and the improvements obtained through this molecular encapsulation.

3.3. China

In this CRP, we have gotten progress mainly in two directions, *i.e.*, the radiolytic syntheses of inorganic nanoparticles and nanostructures in aqueous solutions, and the preparation of inorganic-polymer hybrid microgels by γ-irradiation and surfactant-free emulsion polymerization.

In the first section, for the first time, Prussian blue (PB) nanoparticles (NPs) were successfully synthesized by the partly radiolytic reduction of Fe$^{3+}$ and Fe(CN)$_6^{3-}$ in the presence of poly(N-vinyl pyrrolidine) (PVP) under N$_2$ atmospheres at room temperature. With the increase of the concentration of PVP, the size and the size distribution of the synthesized quasi-spherical PB NPs decreased obviously. Furthermore, it was found that the smaller ones have a larger capacity to Cs$^+$, suggesting that the application of PB NPs in curing thallotoxicosis may decrease the usage of PB for patients to a great extent. Secondly, we found two effective methods to synthesize mesoporous BaSO$_4$ microspheres with a larger pore size by γ-irradiation, which provide connected channels for mass exchange between the inner space of BaSO$_4$ microspheres and the outer solution. These preliminary experiments created favorable conditions for the CrO$_4^{2-}$ ion-exchange experiments. Thirdly, through the controllable radiolytic reduction of Cu$^{2+}$ by β-cyclodextrin, we suggested an interaction mechanism between NPs and β-cyclodextrin, which will favor the usage of CDs-protected NPs in biomedicine.

In the second section, through a series of preliminary experiments, we got a clear picture about the one-step radiolytic preparation of inorganic-poly(methacrylic acid-co-methyl methacrylate) hybrid microgels by surfactant-free emulsion polymerization. Besides, unpurified N-carbamothioyl-methacrylamide was synthesized *via* the methacrylation of thiourea. These created favorable conditions for the one-step synthesis of metal sulfide-poly(methacrylic acid-co-methyl methacrylate) hybrid microgels by γ-irradiation and surfactant-free emulsion polymerization.
3.4. Egypt

Adopting polyvinylpyrrolidone as template macromolecules and acrylic acid (AA) as monomers, at a concentration ranged from .05 to 1.5 %, pH sensitive nano-particle colloids were successfully prepared via template polymerization using gamma radiation in which polymerization of the monomer and self-assembly between the polymer and the template take place simultaneously. The self-assembly was driven by specific interactions between PVP and PAA produced in-situ, leading to PVP/PAAc nano-particles with insoluble inter-polymer complexes. Dynamic light scattering technique was used to indicate size shrinkage and surface charge increase of the PVP/PAAc nanoparticles. Many factors affecting the PVP/PAAc nano-particle size such as irradiation dose rate, exposure dose, irradiation temperature and atmosphere, PVP MWt, and feed composition and concentration were investigated. It was found that the reactant feed composition and irradiation temperatures have a great influence on particle size of the prepared nanogel. The structure and morphology of the nano-particles were characterized by FT-IR, UV, viscometry and AFM methods. The structure stability of the nano-particles was studied at different pH solutions. The nano-particles exhibit excellent pH response. When pH changed from acid to base, the particles’ volume expanded 100 times depending on the irradiation dose at which the nanogel was prepared. The prepared nanogel was loaded with flutamide anticancer drug in the presence of ethanol -water mixture solution and the amount of loaded flutamide was determined. The prepared nano scale polyvinylpyrrolidone/polyacrylic acid bio-polymeric system loaded with flutamide drug is being investigated as anticancer target drug. Also this system will be tested for the treatment of dry-eye-syndrome.

Work Plan
1- Further investigation on the possible applications of the PVP/PAAc colloid nanogel prepared by ionizing radiation as a target drug carrier or in treatment of dry-eye-syndrome will be done.
2- Preparation and characterization of nano silver with antimicrobial applications using natural biopolymer, as a reducing and stabilizing agent will be studied. The influence of different parameters such as natural polymer particle size, concentration of natural polymer, concentration of silver nitrate and irradiation dose, etc will be studied.
3- The study will include radiation synthesis and characterization of electrical and magnetic responsive nano-composite materials such as nano stabilized Fe₃O₄ for biomedical and industrial purposes.

3.5. France

Angiogenesis plays a critical role in both growth and metastasis of tumors. Vascular endothelial growth factor (VEGF) is an endogenous mediator of tumor angiogenesis. Blocking associations of the VEGF with its corresponding receptors (KDR) have become critical for anti-tumor angiogenesis therapy. A cyclo-peptide (CBO-P11), derived from VEGF, able to inhibit angiogenesis was synthesized in our laboratory. We have prepared biocompatible poly (vinylidene fluoride) (PVDF) nanoparticles in order to obtain long circulating drug delivery systems. These particles were characterized and they were found monodisperse with a mean radius of 60 nm. Electron-beam (EB) irradiation was used to activate PVDF nanoparticles. From electron paramagnetic resonance (EPR) measurements, we studied the radical stability in order to optimize the radiografting of acrylic acid (AA). Further functionalization of PVDF-g-PAA nanoparticles
with the cyclo-peptide via a spacer arm was also possible by performing coupling reactions. High resolution magic angle spinning nuclear magnetic resonance (HRMAS NMR) and MALDI mass spectrometry allowed us to follow each chemical step of this peptide immobilization.

3.6. Hungary

Implants play a crucial role in modern medicine. Besides the numerous advantages they provide their use in many cases accompanied by different drawbacks in form of side effects, rejection, inflammation etc. Most of these disadvantages are directly related to the material used for implant fabrication. One way of their elimination (and also the improvement of the properties of the implants) is the use of surface coatings. In addition to the protection function and separation of tissues from the implant material coatings could also deliver drugs and so promote the acceptance of the artificial device and the regeneration of the tissues after the intervention. This project is aimed to develop a drug-eluting porous polymer coating by radiation induced polymerization that can be used in different medical implants.

During the first stage of the project preparation methods have been developed for porous diethylene glycol dimethacrylate (DEGDMA) and composite 2-hydroxyethyl methacrylate (HEMA)/DEGDMA coatings on medical grade 316L and Inconel alloy surfaces by radiation induced polymerization.

Porous polymer DEGDMA drug-eluting coatings have been prepared on metallic alloys by radiation induced polymerization. The required coverage of the metal surface by the monomer mixture was achieved by using ethanol/glycerol solvent mixture. New HEMA/DEGDMA composite polymer layers have also been developed, that can be used as drug delivery coatings on medical implants. They can be prepared either in form of multi-layer HEMA-DEGMA structures by a multi-step irradiation polymerization process or in a single step by using HEMA/DEGDMA monomer mixture with increased DEGDMA content. In the latter case the resulted material contained porous DEGDMA nanoparticles embedded into the HEMA hydrogel matrix.

Raman spectroscopic analysis of the DEGDMA polymerization with irradiation dose showed that intact C=C bonds remain in the polymer even after 100% conversion, indicating the presence of trapped or partially polymerized monomers in the matrix. Their amount decreases with the increase of the DEGDMA concentration in the monomer mixture, which also influences the mechanical properties of the formed polymer.

Based on the CRP we started collaboration with the group of Prof. Kwang-Pill Lee (South Korea) and in 2009 we got a 2-year support from the Hungarian-South Korean Intergovernmental S&T Programme. The aim of our common work is to prepare and characterize functionalized nanocrystalline diamonds by radiation methods.
Work Plan
Optimization of the preparation parameters in order to improve the properties of the drug-eluting layer:
Modification of mechanical properties by changing the composition of the hydrogel;
Improving the adhesion to substrate;
Control of the drug elution properties by changing the porous properties of the hydrogel.
Testing the drug delivery properties of the coating:
Loading mechanisms;
Drug elution tests.

3.7. India

Colloidal metal nanoparticles are of interest in both research and technology, as these possess specific properties not available in isolated molecules or bulk metals. In the last few years, gamma irradiation techniques for generating metal nanoparticles are being explored. The surrounding environment of metal nanoparticles plays a very important role in the optical properties of the nanoparticles. This property along with high sensitivity of longitudinal surface Plasmon band of anisotropic metal nanoparticles towards surrounding environment are useful in application of the metal nanoparticles as sensor. The work being pursued in this CRP is directed towards the following:

Seeded radiolytic synthesis of anisotropic gold nanoparticles in CTAB using gamma radiation
Anisotropic shape was induced in the growth process by controlling the dose rate in presence of structure directing agent cetyl trimethyl ammonium bromide (CTAB) and Au seeds. As the dose rate decreased to 0.8 kGy h\(^{-1}\), the adsorption of Au\(^0\) on growing nanoparticles become slower resulting in formation of short aspect ratio (A.R. 1.5) gold nanorods. The Au nanorods were characterized by UV-Vis spectrometry and TEM microscopy. By decreasing the dose rate the rate of reduction decreases, hence the subsequent growth kinetics becomes slower favoring anisotropic growth.

Seedless synthesis of gold nanorods using gamma radiolysis technique
A growth solution containing Au\(^+\), CTAB, isopropanol, acetone was irradiated at higher dose rate, i.e., 3.4kGy h\(^{-1}\) in absence of any seed particles to obtained Au nanorods of aspect ratio 2.5. Isopropanol reacts with H and OH and acetone reacts with e\(_{aq}^-\) to give isopropyl radical. Isopropyl radical being a mild reducing agent reduces Au\(^+\) to Au\(^0\) and the reaction is slower. The slower reaction helps in formation of Au nanorods.

Synthesis of rectangular plate like gold nanoparticles by combining both radiation and chemical methods
By combining the chemical and radiolytic method gold nanoplates have been synthesized. Ascorbic acid is used to reduce Au\(^{3+}\) to Au\(^+\) and by irradiating this Au\(^+\) solution for small duration of time required amount of seeds are formed in situ which afterwards helps in nanorod growth in presence of excess ascorbic acid. Effect of different experimental parameters on nanoplates formation were studied.
Synthesis of CdSe quantum dots in PVA matrix by radiolytic methods

CdSe nanoparticles were synthesized in PVA matrix by both gamma and electron beam irradiation, where the growth of the nanoparticles and the networking of the PVA polymer took place simultaneously during the irradiations. The effect of dose rate on size of CdSe nanoparticle has been studied.

Ammonia sensor has been developed from silver nano clusters in acrylic acid, which can sense 30-150 ppm ammonia in aqueous solution.

The nanoparticles were characterized by UV-Vis spectrometry XRD technique and TEM microscopy. Hence radiation method is very effective for the synthesis of metal nanoparticles. Since reducing species are generated in-situ, the products are free from the contaminations arising from external chemical reducing species used in chemical method.

Workplan

Further study on shape selective synthesis of metal and semiconductor nanoparticles. Detection and estimation of important biological and other chemical analytes.

3.8. Iran

Poly(Lactic acid) (PLA) is one of the most popular biopolymers as a polymer matrix of choice for many researchers. It is aliphatic thermoplastic polyester produced from renewable natural resources. The high melting temperature, good process ability and mechanical properties of PLA along with its biodegradability and biocompatibility makes it a preferred candidate for biomedical and pharmaceutical applications such as prosthetic implants, controlled release systems and three dimensional scaffolds. However its properties can be further enhanced by introducing inorganic additives such as layered silicate, hydroxyapatite and metal nanoparticles to it to form a hybrid material. The goal of this research work is to improve the performance of PLA films by the incorporation of nanosized particles. Poly (Lactic acid)/organically modified layered silicate films were prepared by solution casting method. The nanocomposite films were irradiated in a gamma cell irradiator facility with source of Co$^{60}$ at 30 kGy. The effect of gamma irradiation on mechanical properties of the neat PLA and nanocomposites was evaluated by data obtained from tensile testing measurements. The tensile strength of the irradiated PLA films increased with the addition of 1 wt% Triallyl Cyanurate (TAC) indicating crosslink formation at low dose of 30 kGy. Significant ductile behavior was observed in the PLA nanocomposites containing 4 pph of nanoclay. Incorporation of nanoclay particles in the PLA matrix stimulated crystal growth as demonstrated by differential scanning calorimetry (DSC). The morphology of the nanocomposites characterized by transmission electron microscopy (TEM) and X ray diffraction (XRD) revealed an exfoliated morphology in the PLA nanocomposite films containing 4 pph of nanoclay. Only very small changes were observed in the chemical structure of the irradiated samples as it was investigated by Fourier transform infrared (FTIR) spectroscopy. The results obtained from the enzymatic degradation test showed that weight loss of the pristine PLA and its nanocomposites depends on crystallinity and morphology of the samples. The preliminary results on stress-strain test of the PLA/HAP showed that mechanical properties were dependent on the composition and morphology of the prepared samples. The ductility of the irradiated PLA nanocomposites containing 10 pph of HAP increased 3 times compared to that of the neat irradiated PLA.
**Work plan**
Solution casting of poly (Lactic acid)/hydroxyapatite nanocomposite with various compositions
Irradiation of nanocomposites with γ-ray doses of 25 and 30 kGy.
Characterization of nanocomposite films by FTIR, DSC, Stress-Strain, XRD and TEM tests.
Enzymatic degradation test of the nanocomposites.

3.9. **Italy**

In the last two decades we are witnessing to an increased interest in nanomaterials for medicine with the aim of radically improving current therapies and diagnostic modalities. The research, though, has often progressed in a very fast but fragmented and not coordinated way both in academia and industry. Several different nanomaterial systems have been proposed including colloidal systems and liposomes, quantum dots (QDs), organic and inorganic dendrimers, viral and virus-like nanoparticles, polymeric vesicles and solid lipid nanoparticles. All the proposed approaches have specific advantages, but also shortcomings and limitations, mostly related to the huge gap between the in-vitro and the in-vivo evaluation tests results and to the limited possibility of transforming many of the inventions into robust, economically viable technology platforms.

High energy radiation processing has demonstrated its potential for the production of hydrogel nanoparticles already in the late ‘90s, owing to the pioneeristic work of Prof. Rosiak and collaborators, but since then no adequate efforts have been spent in developing a viable industrial technology to produce radiation engineered multi-functional nanoparticles with the required properties for application in drug delivery. To this goal a multidisciplinary approach that embraces radiation chemistry, materials science, process technology, biochemistry and medicine is required. The research activity under the present CRP is providing evidence of the possibility of generating biocompatible multifunctional nanogels with dimensions varying in a range of tens to hundred of nanometers, using existing industrial-type accelerators and set-ups. The produced hydrogel nanoparticles have been characterized in terms of molecular structure, particle size and size distribution, stability, ability to redisperse in aqueous solutions and buffers from the dry physical form. Covalent attachment of a fluorescent dye has been achieved, thus allowing visualization of the nanogels in-vitro cell cultures. The simultaneous PVP crosslinking and grafting of a primary amine carrying group monomer is providing pH responsiveness and extra-stability in solution at neutral and alkaline pHs. In the further development of the research the functional groups grafted to the networks will be used to attach selected targeting ligands. Furthermore, GdIII-complexes will be incorporated as contrast agents to enable in vivo biodistribution studies through NMR imaging.

3.10. **Japan**

Introduction of functional regions in nanometer scale in polymeric films using γ-rays, EB, and ion beams are proposed. We have been attempting two approaches to built nano-scale functional domains in polymer substrates: 1) Radiation-induced grafting to transfer nano-scale polymer crystalline structures (morphology), acting as a nano-template, to nano-scale graft polymer regions. The obtained polymers with nano structures can be applied to high performance polymer membranes. 2) Fabrication of nanopores and functional domains in engineering plastic films using ion beams, which deposit the energy in very narrow region of polymer films. The followings are the outputs of our research project in the first year.
1) Hydrophilic grafting polymers are introduced into hydrophobic fluorinated polymers, cross-linked PTFE (cPTFE) and aromatic hydrocarbon polymer, poly(ether ether ketone (PEEK), which is known to have lamella and crystallite in the polymer films. Then, the hierarchical structures of graft domains are analyzed by SAXS and SANS experiments. From these analyses, the different structures and the different formation of graft domains were observed in fluorinated and hydrocarbon polymer substrates. In the case of cPTFE, the grafted domain, working as an ion channel, grew as covering the crystallite and the size of domain seems to be similar to that of crystallite. On the other hand, the PEEK PEM has smaller domain size and it seems to grow independently on the crystalline phase of PEEK substrate.

2) For nanofabrication of polymer films using heavy ion beams, we took notice of energy distribution in radial direction, which is perpendicular to ion trajectory. For penumbra, we re-estimated effective radius of penumbra, in which radiation induced grafting took place, for several different ion beams. We observed the different diameters of the ion channels consisting of graft polymers. The channel sizes are quite in good agreement with the size of effective penumbra which possess the absorption doses more than 1 kGy. In the coming year, we continue the both projects. For the nano transfer to polymer films using EB and g-rays, we control the shape and size of crystallites in polymer substrates and check the shapes and sizes of graft domains. For ion beam nano fabrication, we plan to make nano pore formation of fluorinated polymers such as PVDF. These films should be applied for selective separation membranes, reactors including catalysts for pharmaceutical synthesis and DNA recognition for biosensors.

3.11. Malaysia

The use of microemulsion in the development of nanosized gel based on polyethylene glycol diacrylate (PEGDA) and acrylated palm oil (APO) is demonstrated. PEGDA was solubilized in n-heptane with use of AOT at 0.15M concentration to form reverse micelles, while APO was solubilized with SDS in water to form direct micelles. Both of these systems were depicted by means of ternary phase diagram. These micelles were than irradiated at 1,3,5,10,15 and 30kGy using gamma irradiation or EB to crosslink the entrapped polymer in the micelles. Ionizing radiation was imparted to the emulsions to generate crosslinking reactions in the micelles formed. The nanosized gel was evaluated in terms of particle diameter using dynamic light scattering and the images of the nanosized gel were studied using transmission electron microscopy (TEM). Results show that the size, charge and shape of the particles are influenced by concentration of surfactants and radiation dose. This study showed that this method can be utilized to produce nanosized gel. Future work include the attachment of functional group to the nano sized gel, loading of drug such as curcumin and further characterization using dynamic light scattering.

3.12. Poland

Radiation induced method of nanogels synthesis elaborated at Institute of Applied Radiation Chemistry, Technical University of Lodz, has been used for synthesis of multi-component nanogels based on polymer complexes. These polymolecular structures can potentially serve as delivery systems of drugs with poor intrinsic water solubility or hydrophobic peptides. Irradiation, in
conditions promoting intermolecular cross-linking, of inter-polymer complexes solutions led to the fixation of a complex structure by covalent bonds. In consequence, irradiated complexes shows pronounced response to solution pH and ionic strength. They are also more resistance against radical induced degradation than origin macromolecules. Mechanism of radiation induced intramolecular cross-linking has been studied by Monte Carlo simulation using cooperative motion algorithm (CMA) and pulse radiolysis. Simulations, in good agreement with experiments, showed dispersive kinetics of the process. Simulations of simple model cases (fixed distance between radicals, formation of loops, radical transfer), which are difficult or impossible to investigate experimentally, broaden the understanding of the importance of elementary processes which contribute to kinetics of recombination of randomly distributed radicals on long chains. It has been shown that the main factor determining the radical recombination is the distance between them along the chain which strongly favors nearest-neighbor reaction.

In the third year of realization, the following tasks within the Project will be continued:

**Radiation-induced synthesis of polymeric nanogels:**
Nanogels are being synthesized by the pulse irradiation method elaborated at Institute of Applied Radiation Chemistry. Beside determination of nanogels properties mechanism of intramolecular cross-linking will be examined. Both Monte-Carlo simulations and experimental techniques will be used for analysis of changes in nanogels internal structure. Synthesized nanogels will be studied by viscometry, gel permeation chromatography, static and dynamic laser light scattering, zeta potential and laser diffraction.

**Formation and preservation of inverse emulsion of polymers:**
Inverse emulsions containing nano- and microdroplets of aqueous polymer solution will be fixed by radiation-induced grafting and cross-linking. Influence of substrate composition, including kind of organic phase, kind and amount of dispersing agents, water to oil ratio, kind and concentration of a polymer as well as irradiation conditions on the properties of products will be studied. Product analysis will be based on FT-IR and UV-Vis spectroscopy, viscometry, gel permeation chromatography, static and dynamic laser light scattering, zeta potential and laser diffraction as well as scanning electron microscopy.

**Application of radiation technique for synthesizing nano- and microlayers of thermoresponsive polymers and gels:**
Ionizing radiation will be used to graft monomers on a range of polymer supports in order to obtain thin (micro- or nanometer-range) thermoresponsive layers used for cell layer engineering. Thermocontrolled scaffolds, based for example on poly(vinyl methyl ether) will be examined for its applicability in cell cultivation. Products will be characterized by FT-IR (HATR mode) and UV-Vis spectroscopy, scanning electron microscopy, AFM, ellipsometry and contact angle measurements.

3.13. **Republic of Korea**

An exciting new trend in biotechnology is to develop novel functional nanomaterials that can used for application like imaging diagnostics, sensing, and controlled drug delivery. In recent years,
fabrication of nanomaterials with hollow interiors has received increasing attention in nanoscience and nanotechnology owing to their potential applications in photonic devices, drug delivery, material encapsulation, ionic intercalation, surface functionalization, nanocatalysts, and membrane nanoreactors, etc. In response to the technological requirements, there have been numerous methods in place. These methods can be broadly divided into two types: hard template and soft template approaches, where solid materials such as anodic alumina membranes or mesoporous silicas and soft templating materials such as ionic or non-ionic surfactants, polymers, or organic ligands have been utilized as structural guiding matters or reagents. Recently, conducting polymer (CP) nanostructures such as nanotubes, nanodiscs and hollow spherical nanoshells have received interest because of their physico-chemical properties and potential applications. A variety of synthetic approaches which include interfacial polymerization etc., have been developed for the preparation of CP nanostructures. In recent years, there has been increasing interest in the fabrication of composite hollow spheres consisting of CP as the support matrix and metal nanoparticles (MNPs) or other catalyst particles as the other component. To the best of our knowledge, reports on radiation assisted synthesis of CP based composite nanocapsules are scarce.

We report the preparation of hollow spherical nanocapsules (HSNC) and subsequent modification onto hollow spherical composite nanocapsules (HSCNC) by loading MNPs into the HSNC(PPy) through γ-radiation. The HSCNC is designated as HSCNC(PPy/MNP) in terms of the shell (PPy) and loaded metal nanoparticles (MNPs). The preparation of HSCNC(PPy/MNP) involves steps as detailed in Scheme 1. In this case, γ-irradiation was used for loading MNPs onto HSNC(PPy). Also, we have utilized the above methodology for the preparation of HSNC based on PANI using γ-irradiation. We have prepared PANI based HSNC(PANI) and HSCNC(PANI/MNP). HSCNC(PPy/MNP) and HSCNC(PANI/MNP) were independently prepared by different approaches to bring out the effectiveness of γ-irradiation in these preparations. γ-irradiation technique has been previously used to generate nanoscale metals and nanocomposites. Moreover, γ-irradiation technique has several advantages. Importantly, γ-irradiation method can produce pure or clean materials without impurities. It is possible to control the size of particles by proper selection of radiation dose etc.

Trial studies have also been done for the second phase of RCM to develop functional electrospun polymer nanofibers (F-ESPNF) through radiation induced processes. Functional nanostructures are currently the subject of increasing interest. Importantly, it is fairly easy to include functional properties to the ESPNF by incorporating additives during the electrospinning process. Various types of additives could be incorporated into the ESPNF. Large surface area, excellent moisture/gas transport, extremely low air permeability and unique porous structures of ESPNF are effectively used to improve current technology and applications. Our experimental findings give hopes that ESPNF could be suitably modified by radiation to develop F-ESPNF. It is planned to utilize HSCNC(PANI/MNP), HSCNC(PPy/MNP) and F-ESPNF for biosensing, bielectrocatalytic and drug release applications.

3.14. Serbia

The research activities in Serbia were related to radiolytic synthesis of polymer based nanocomposites with noble metal nanoparticles. The aim of the work is systematically developing synthetic strategies for incorporation of nano-Ag in hydrogel networks by gamma irradiation, using
liquid filled cavities in hydrogels as nanoreactors (template synthesis), and exploring favourable characteristics of radiation technology for nanoscale engineering of materials especially for biomedical application. Advantages of radiolytic method are numerous, reactions occur under the standard conditions, no formation of by-products, no need for any catalyst, enable crosslinking of polymers, suitably for reduction of metal ions and finally possibility of synthesis and sterilization in one technological step.

The chosen hydrogels, being previously synthesized or crosslinked by gamma irradiation, are suitable for various applications in reconstructive surgery, including wound dressing, tissue expanders etc. Nano-Ag is successfully incorporated in hydrogel matrix such as PVA, PVP, poly(HEMA-co-IA) copolymer and PNIPAA. Obtained results indicated that gamma irradiation is suitable for in situ generation of Ag nanoparticles in investigated hydrogel matrix by radiolytic products of water. X-ray diffraction analysis confirms the fcc structure of Ag nanoparticles. Swelling properties of synthesized hydrogels, pure and Ag/hydrogel nanocomposites, investigated in the SBF (simulated body fluid) solution at 37 °C exhibits that Ag/hydrogel nanocomposite systems have higher equilibrium swelling compared with pure hydrogel. The release of silver Ag from nanocomposite systems is continuous during long period of time which means that investigated hydrogel nanosystems meet criteria of sustained, steady supply of active silver. Further work on antibacterial properties of these materials is in progress.

**Work Plan**

During the second stage of realization of the Project we would like to continue studies on the following topics:

Second year
Investigation of antibacterial properties of radiolytically synthesized Ag/hydrogel nanocomposites accordingly to standard protocols.

Third year
Synthesis of Au nanoparticles in previously obtained Ag-hydrogel nanocomposites by gamma irradiation. Investigation of biological potential of synthesized AuAg-hydrogel nanocomposites.

3.15. Thailand

It is well known that polymer micelles have unique core–shell architecture that composed of hydrophobic segments as internal core and hydrophilic segments as surrounding corona in aqueous medium. The hydrophobic core provides a loading space for water-insoluble drugs, whereas the modification of hydrophilic shell affects pharmacokinetic behavior. Additionally, the nano-scaled polymer micelles exhibit many advantages for the use of drug delivery carriers, such as prolonged circulation; tumor localization by enhanced permeability and retention (EPR) effect; and the controlled drug release by using stimuli-sensitive copolymers.

Chitosan have been in recent years extensively researched as a primary material in forming carriers and widely used in pharmaceutical and medical areas. The modification of chitosan to form self-assembly chitosan nanoparticle using chemical synthesis has been reported in many protocols. The modification of chitosan using conjugating agents is the most widely used. The hydrophobic and hydrophilic molecules are commonly functionalized onto chitosan via reactive amino and hydroxyl group at C-2 and C-6 position to from amide and ester linkage. In this way, not only loss of
potential cationic functions of chitosan but also carrying out with several steps in preparation has to be considered.

Therefore, study on systematic condition in preparing self-assembly chitosan nanoparticle has been being in our interest to achieve a novel protocol beside conventional one. In the present works, self-assembly chitosan nanoparticle (CsNP) has been synthesized via radiolytic methodology using gamma irradiation. The systematic conditions in preparation using among simultaneous radiation synthesis, direct irradiation and conventionally chemical modification have been studied. Chitosan nanoparticle was modified using hydrophobic core of deoxycholic acid (DC) and stearyl methacrylate (SMA) and the hydrophilic shell of polyethylene glycol monomethacrylate (PEG). The hydrophobic/hydrophilic CsNP was prepared to use as a drug carrier molecule. In addition, SMA-CsNP was also applied by conjugating with pyperidine, hindered amine light stabilizer function, to achieve a bio-based additive for biomedical plastic.

Work plan for the next 18 months will be (i) extensively studied on radiation synthesis of hydrophobic/hydrophilic self-assembly CsNP (e.g. effect of solvent and monomer concentration) and (ii) radiation synthesis of water-soluble chitosan and PEG stabilized gold nanoparticle: an approach to radio-therapeutic substance.

3.16. Turkey

Radiation-induced crosslinking of poly(vinyl pyrrolidone) chains in aqueous solutions was elaborated by considering the solution thermodynamics in order to control the coil dimensions of polymer chains before crosslinking. Gradual approach to theta conditions in acetone/water solutions provided the control of PVP coil sizes within 5-10 nm ranges. E-beam and gamma irradiation of dilute PVP solutions in the range of 5-15 kGy range was found to be sufficient to obtain nanogels. Gel Permeation Chromatography and Scanning Electron Microscopy techniques in addition to Nano/Zeta Sizer and Atomic Force Microscopy techniques were used for the characterization of nanogels. Nanoscale grafting of some stimuli-responsive polymers such as poly(N-iso-propylacrylamide) and poly(acrylic acid) onto cellulose was achieved by the use of reversible addition-fragmentation chain transfer technique. By controlling parameters such as total absorbed dose, dose rate and the ratio of monomer to chain transfer agent it has been shown that it is possible to have very close control of the molecular weight and distribution of grafted chain lengths. The control of molecular weights was in the range of 8000-90000 for the grafted chains with a polydispersity of 1.1-1.2.

3.17. USA

We are using RAFT polymerization to synthesize smart polymer nanocarriers for intracellular delivery of protein, peptide and nucleic acid drugs. Smart T- and pH-responsive polymer nanocarriers have been RAFT synthesized for enhanced intracellular delivery of biomolecular drugs such as peptides, proteins and nucleic acid drugs.

In the coming program period we plan to synthesize these carriers using radiation to initiate the RAFT polymerizations. In this way we will avoid the need to add free radical initiators to initiate this polymerization, yielding a purer polymer-drug nanocarrier.
4. CONCLUSIONS

After reviewing the Participating Countries Reports according to the CRPs specific objectives five different areas of research have been identified as nanoparticles, nanoporous membranes, nanocarriers, nanocomposites, surface modification at the nanoscale:

4.1. Nanoparticles (metallic, inorganic, clusters)

By in situ gamma-radiation synthesis, Ag spherical nanoparticles have been successfully obtained in controlled sizes inside a previously radio-crosslinked polymer gel made of PVP, PVA, PNIPAM, P(HEMA-co-IA). Ag NPs have also been immobilized onto PVA radio-grafted single walled and multi-walled CNTs.

Quantum dots embedded in PVA matrix (film) have been simultaneously synthesized by gamma and e-beam irradiation. Shape selective synthesis of noble metal NPs by gamma-radiation: nanorods, nanoplates. Possible application in nano-photonic field and bio-chemical sensors.

Other kind of spherical NPs have been obtained by first, nano-emulsion polymerization of VDF gaseous monomer in aqueous solution and secondly, the obtained latex of PVDF NPs have been radiografted with PAA in situ by gamma-irradiation leading to fully organic core-shell NPs.

Some inorganic NPs made of Prussian blue and barium sulfate compounds have been synthesized by gamma-radiation, whose morphology and size can be accurately controlled. These NPs have been used for metal ion exchange or adsorption, specifically for Tl+, Cs+ and CrO42-.

Cu NPs were produced via reduction of Cu2+ in the presence of cyclodextrins by gamma-radiation. An interaction mechanism between Cu NPs and cyclodextrins was suggested.

The above mentioned nanoparticles were intended to be applied in medical imaging, sensors, antibacterial applications, drug-targeting, nanosorbents and nano-medicine.

4.2. Nanostructured membranes

The effective radius of penumbra induced by heavy ion beams irradiation has been re-estimated. Ion channels consisting of graft polymers with different diameters have been generated. The prepared membranes have been successfully tested in PEM fuel cells.

Nano-scale graft domains were introduced in fluorinated polymer substrates. In the case of crosslinked PTFE, the grafted domain, working as an ion channel, has grown to cover the crystallites.

Energy distribution for fabrication of nano-pores in engineering plastic films using ion beams was estimated by taking in account the energy distribution along the radial direction. The heavily damaged area within 1 nm was used for the formation of nano-pores in fluorinated polymer films.
Drug-eluting HEMA/DEGDMA coating has been synthesized with tailored nanostructure by radiation polymerization for use in medical implants. The adhesion of the coating on the substrate was improved by use of hydrophilic amorphous carbon layer prepared by radio-frequency chemical vapor deposition.

4.3. Nanocarriers

A number of different natural and synthetic polymers have been used to obtain potential nanocarriers for drug delivery
- Natural polymers: Albumine, Chitosan, Palm oil acrylates
- Synthetic polymers: PEGDA, PAA, PVP, PVDF, PVA, PPy+PANI (conductive polymer)

Control of size (10-300nm) and shape (hollow spheres, hard sphere, soft gels) has been demonstrated.

Two different approaches have been followed to control the size and shape of the nanocarriers, one consists on the control of thermodynamics of polymers in aqueous solutions and the other one is based on the synthesis with hard/soft-templates.

*Synthesis with hard/soft-templates*

Synthesis of hollow spherical nanocapsules using SiO$_2$ NPs as template.
- Synthesis of PEGDA and Acrylate Palm Oil nanogels in inverse or direct microemulsion.

*Synthesis in aqueous solution*

On the ground of previous findings, new approaches/methodologies and characterization techniques have been established for the radiation synthesis of the PVP-based nanogels.

The mechanism of nanogel formation has been examined. The conformation of the polymer in dilute/semi-dilute solution and the distance between particles and macroradicals has been proved to be a controlling factor for nanogel formation. Interpolymer complexation phenomena has been followed to improve functionality. Stabilization of IPCs by nanogel synthesis has been presented as well as pH sensitivity.

Improved control of particle size has been shown to be achieved by both the initial polymer molecular weight and by the control of the thermodynamical properties of aqueous polymer solution, e.g. by approaching theta conditions.

Radio-synthesis of protein-based nanoparticles has been also achieved.

Introduced multifunctional groups for improved stability, redispersibility from the dry state and affinity for some specific drugs or biological molecules of interest.

Small anti-angiogenic cyclo-peptides have been immobilized onto radio-grafted PVDF NPs to target especially cancer cell.
4.4. **Surface modification**

Procedures for RAFT-mediated and radiation induced grafting of stimuli-responsive polymers such as PNIPAM and PAA onto cellulose has been established providing a close control of graft chain length and molecular weight distribution.

4.5. **Nanocomposites**

Radiation synthesis of multi-functional nanofillers with light stabilizing and compatibilizing properties, based on biodegradable materials (notably Chitosan) has been achieved.

Composite nanoparticles have been produced by radiation induced oxidative polymerisation of aniline or pyrol onto silica nanoparticles surfaces in the presence of gold or platinium and ruthenium salts for biosensing and biocatalytic applications.

By in situ gamma-radiation synthesis, Ag spherical nanoparticles have been incorporated inside a previously radio-crosslinked polymer gels made of PVP, PVA, PNIPAAM, P(HEMA-co-IA). Those flexible materials show the continuous release of Ag⁺ allowing to explore further their antibacterial activity.

PLA/organically modified layered silicate and PLA/hydroxyapatite nanocomposites, having exfoliated morphology, with improved ductility and thermal stability were prepared. It was found that enzymatic degradation rate of nonirradiated and irradiated PLA organoclay nanocomposites depends on both their morphology and crystallinity.

4.6. **Collaborative work**

- **Malaysia-Thailand:** exchange of samples for nanoparticles characterization
- **Malaysia-Poland:** characterization of nanogels using AFFF
- **Poland-Italy:** access to E-Beam irradiation facility
- **Poland-Argentina:** access to E-Beam irradiation facility for protein-based nanoparticles production and sterilization.
- **Brazil-Argentina:** synthesis and characterization of protein-based nanoparticles
- **France-Argentina:** swift heavy ions irradiation of polymeric films for membrane production
- **France-Korea rep. of:** synthesis of hollow spheres also containing nanoparticles
- **Serbia-Turkey:** exchange of samples for characterization.
- **Serbia-France:** exchange of samples for characterization
- **Korea Rep of.-Hungary:** Preparation and characterization of functional nanoparticles
- **Turkey-Italy:** student exchange
- **Italy-Hungary:** exchange of samples for characterization
5. RECOMMENDATIONS

The participants made the following recommendations regarding the future research:

5.1. Nanoparticles (metallic, inorganic, clusters)

Test the potential of radiolytically synthesized metal and semi-conducting nanoparticles in sensing biologically important molecules (proteins, peroxides).

Improve performance of nanoparticles in the removal of hazardous substances, such as Cs$^+$ and Tl$^+$, from the environment.

5.2. Nanocarriers

Provide further evidence that nanogels and other organic and inorganic nanostructures can be potentially used for drug encapsulation and controlled delivery.

Improve the control of the particle size of nanocarriers for cancer therapy in order to enhance the drug delivery efficiency e.g. through the enhanced permeation and retention (EPR) effect.

Perform biological evaluation of unloaded and loaded nanocarriers through in vitro cyto-toxicity, in vivo toxicity and clinical trials. In the framework of this CRP, in vitro biological experiments are expected. Contacts with labs which can offer opportunity for in-vivo testing is encouraged.

Enlarge the protein-base nanocarrier platform through the recourse to different proteins. Improve the properties of nanogels (functionalisation, size stability, composition, etc.) to enhance drug loading ability and provide targeting functions.

Improve drug delivery devices with NMR contrast agents to enable nanocarrier tracking in cells.

Address biomolecular ligands attachment on nanocarriers for active targeting.

Develop surfactant-free radical polymerization methodologies to obtain nanogels and avoid difficulties in product purification.

Consider the development of self-assembled nanocarriers based on natural polymers.

5.3. Nanostructured membranes

Control shape and size of crystallites in polymer substrates in order to prepare the graft polymer regions (ion channels) in PEM fuel cell.

5.4. Surface modification

Improve radiation induced polymerization/grafting of nano-/microlayers of thermal responsive polymers or gels for cell cultivation.
Develop simultaneous radiation-induced graft polymerization processes of track-etched membranes for biocatalytic applications through enzymes immobilization.

5.5. **Nanocomposites**

Control of morphology and crosslink density of PLA/HAP nanocomposites in order to gain higher mechanical properties and thermal stability.

Consider the prevention of uncontrolled leaching/extraction of silver nanoparticles from the silver/hydrogel nanocomposites.

Assess the performance of radiation stabilized chitosan-based nanofiller blended with biomedical plastic (i.e. PLA).

5.6. **Venue of the 3rd RCM**

It is highly recommended by the Participants of the meeting that IAEA should organize the 3rd RCM in Poland, preferentially in October 2012 in conjunction with IRaP 2012.

6. **LIST OF PUBLICATIONS BY PARTICIPANTS**

BARTOSZEK N, P. ULAŃSKI, J. M. ROSIAK; Free-radical reactions involving macromolecules as studied by pulse radiolysis - factors influencing reaction rate constants, 4th European Young Investigators Conference, Collegium Polonicum, Słubice (Poland); 18 - 21 June 2009

BARTOSZEK B, P. ULAŃSKI, J. M. ROSIAK; Radioliza impulsowa jako metoda do wyznaczania stałych szybkości propagacji w polimeryzacji rodnikowej. 52. Zjazd Polskiego Towarzystwa Chemicznego oraz Stowarzyszenia Inżynierów i Techników Przemysłu Chemicznego, Łódź 12-16 September 2009


KADLUBOWSKI S, R. CZECHOWSKA-BISKUP, P. ULAŃSKI, J. M. ROSIAK; Inicjowana radiacyjnie syntezę nanażeli polimerowych, III Krajowa Konferencja Nanotechnologii - nano2009, Warszawa (Poland); 22-26 June 2009

KRKLJES, A., KACAREVIC-POPOVIC, Z., NEDELJKOVIC, J., “Radiolytic synthesis and Characterization of Thermoresponsive Ag/PNIPA Hydrogel Nanocomposites”, Second


ROKITIA B., P. KOMOROWSKI, P. ULANSKI, B. WALKOWIAK, J. M. ROSIAK; Sonolysis of DNA from calf thymus in aqueous solution, 4th European Young Investigators Conference, Collegium Polonicum, Slubice (Poland); 18 - 21 June 2009

ROKITIA B., P. KOMOROWSKI, P. ULAŃSKI, B.WALKOWIAK, J.M. ROSIAK; Wpływ ultradźwięków na kwas deoksyrybonukleinowy - DNA, 52. Zjazd Polskiego Towarzystwa Chemicznego oraz Stowarzyszenia Inżynierów i Techników Przemysłu Chemicznego, Łódź 12-16 September 2009


ULAŃSKI P.; Radiation chemistry of polymers - some opportunities and trends, Radiation Chemistry in the 21st Century A Visionary Meeting, University of Notre Dame, Notre Dame,Indiana, USA, 12 – 15 July 2009

REPORTS BY PARTICIPANTS IN THE COORDINATED RESEARCH PROJECT
RADIATION SYNTHESIS OF FUNCTIONAL NANOPARTICLES FOR IMAGING, SENSING AND DRUG DELIVERY APPLICATIONS

M. Grasselli, S. Soto Espinoza, V. Risso, E. Pawlak, E. E. Smolko; ARGENTINA

1. INTRODUCTION

Ionizing radiation has been industrially used for many years in polymer processing. The two main streams of applications belong to crosslinking and degradation reactions. More sophisticated commercial products are achieved under development such as hydrogel wound dressing, membranes and sensors [1].

Radiation technology can be used to generate polymer microparticles by precipitation polymerization with a very narrow particle size distribution. Additive-free initiation and easy process control are the main advantage of this technology. In this way, methacrylate-based microspheres can be prepared by radiation-induced polymerization of diethyleneglycol dimethacrylate (DEGDMA) and others cross linkers in organic solvent. Particle diameter can be achieved, from the range 0.8 to 8 µm, by selection of the appropriate organic solvent [2,3]. Tailor-made microspheres were further developed by copolymerization of DEGDMA with reactive monomers, such as glycidyl methacrylate, or particular monomers to rich functional microspheres [4,5], however lower-diameter particles still have not been achieved by this technique.

Small polymeric particles can be also reached by ionizing radiation technology, but via another strategy. Soluble synthetic polymers can be intramolecularly cross linked by quantum-ray, creating nanogels, which was first reported by Ulanski et al. [6,7]. Authors managed irradiation conditions to generate intramolecular crosslinking onto soluble polymer molecules in random coil conformation.

Many proteins are also soluble macromolecules; however, most of them have a very compact and defined three dimensional structure, which are known as globular proteins. The effect of gamma rays onto proteins was study for many years, mainly in features related to degradation. Proteins, as raw material, have been recently used for preparation of nanogels from gelatin, using ionizing techniques [8]. Gelatin comes from the hydrolysis of collagen, a fibrous and insoluble protein. Authors have been studying the changes in the intrinsic volume of these protein nanoparticles. However the first report of nanoparticle synthesis from proteins using radiation technology was done by Furusawa [9]. In both cases, row material was gelatin with average molecular weight of about 100 kDa, a mixture of polypeptides without a defined three-dimensional structure. As a consequence of lack of a defined conformation in solution, this protein is closer to synthetic polymers to enzymes and/or functional globular proteins.

In the present report we describe nanoparticle synthesis by ionizing radiation from globular proteins and methacrylate monomers. Dynamic light scattering and other spectroscopic methods were performed to characterize this new material.
2. MATERIALS AND METHODS

Bovine serum albumin, Fraction V (BSA), diethyleneglycol dimethacrylate (DEGDMA), and glutaraldehyde solution were obtained from Sigma (BA, Argentina).

BSA was dissolved in buffer phosphate 30 mM (BP) pH 7. Different amount of modifiers were added dropwise onto the protein solution at 5 ºC under constant stirring. BSA solutions were irradiated with gamma-ray from a $^{60}$Co source (PISI CNEA Ezeiza) at a dose rate lower than 1 kGy/h at 5-10 ºC. After the irradiation, clear or slickly opaque solutions were observed in the samples. Protein solutions were diluted to a suitable concentration with BP pH 7 for analysis. Fluorescein isothiocyanate (FITC) was used to label BSA with fluorescein according to the method developed by Heuck [10]. Chemical cross-linking was performed by addition of glutaraldehyde solution (final concentration 2.6 %) to achieve particle. All other reagents were of analytical grade and used as received.

Particle size was determined by dynamic light scattering (DLS) at 25 ºC by measuring the autocorrelation function at a 90-degree scattering angle in a 90Plus/Bi-MAS particle size analyzer. Circular Dicroism (CD) measurements were carried out at 20 ºC on a Jasco 810 spectropolarimeter. BSA was subjected to thermal unfolding between 0 and 90 ºC with a heating rate of 2 ºC/min. Secondary structure break down was followed by measuring ellipticity at 220 nm, using the above-described spectropolarimeter and a 1.0-cm cell. UV-absorption spectra (240–340 nm, 0.1 nm sampling interval, 20 nm/s) were obtained with a Jasco V-550 spectrophotometer (Jasco Corporation, Japan). Fourth-derivative of the spectra was calculated applying two successive cycles of second order derivation. Fluorescence measurements were recorded in NanoDrop 3300 Fluorospectrometer. SEM pictures were done in electronic microscope JEOL JSM 5600 LV. For TEM analysis negative stain micrographs was performed with Uranyl Acetate stain with a Zeiss EM 109 Turbo transmission electron microscope.

3. RESULTS AND DISCUSSION

In order to synthesize nano/micro particles we analyzed structured biopolymers usefulness, such as globular proteins as ‘nano seed’ to produce particles. In this way, aqueous solutions of protein and DEGDMA were irradiated. In addition to water, ethanol was added as cosolvent in order to improve monomer solubilization. In Table 1 and 2 are described the composition of different mixtures studied. In the first experiment were irradiated different quantities of DEGDMA maintaining constant a BSA amount, as it is detailed in Table 1, dissolved in PB with ethanol added (40 % v/v). Mixtures were irradiated in a $^{60}$Co gamma irradiation source at 10 kGy and temperature was kept at 0-5 ºC during irradiation.
TABLE 1. BSA AND DEGDMA AMOUNTS DISSOLVED IN BUFFER WITH ETHANOL (40 % V/V) AND IRRADIATED FOR MICROSPHERES SYNTHESIS (TOTAL VOL: 1.5 ML)

<table>
<thead>
<tr>
<th>Sample</th>
<th>BSA (µmol)</th>
<th>DEGDMA (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.7</td>
<td>45</td>
</tr>
<tr>
<td>A2</td>
<td>0.7</td>
<td>90</td>
</tr>
<tr>
<td>A3</td>
<td>0.7</td>
<td>135</td>
</tr>
<tr>
<td>A4</td>
<td>0.7</td>
<td>180</td>
</tr>
</tbody>
</table>

**FIG. 1.** SEM pictures of BSA/DEGDMA microspheres synthesized according Table 1. (Top left: sample A1; Top right: sample A2; Bottom left: sample A3 and Bottom right: sample A4).

With the exception of sample A1, particulate suspensions are yielded after irradiation. Scanning microscopy showed increments in the particle size, up to approximately 200 nm, upon increasing the addition quantities of DEGDMA (Fig. 1), as it can be expected. In the case of varying protein amount and keeping constant the DEGDMA quantity, as it is described in Table 2, all samples reached white dispersions after irradiation. After recovered by centrifugation and washed several times with water and ethanol and dried by lyophilization, SEM pictures were performed.
TABLE 2. BSA AND DEGDMA AMOUNTS DISSOLVED IN BUFFER WITH ETHANOL (40 % V/V) AND IRRADIATED FOR MICROSPHERES SYNTHESIS (TOTAL VOL: 1.5 ML)

<table>
<thead>
<tr>
<th>Sample</th>
<th>BSA (μmol)</th>
<th>DEGDMA (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.23</td>
<td>225</td>
</tr>
<tr>
<td>B2</td>
<td>0.46</td>
<td>225</td>
</tr>
<tr>
<td>B3</td>
<td>0.58</td>
<td>225</td>
</tr>
<tr>
<td>B4</td>
<td>0.7</td>
<td>225</td>
</tr>
</tbody>
</table>

FIG. 2. SEM pictures of BSA/DEGDMA microspheres synthesized according Table 2 (Top left: sample B1; Top right: sample B2; Bottom left: sample B3 and Bottom right: sample B4).

From SEM pictures we can assert that for solutions prepared with increasing quantities of BSA, also yield particle with higher sizes, however, the effect is less intense than in the previous case. Thus, protein had a more complex behavior than can initially be speculated.

Having in mind that globular proteins are macromolecules very sensitive to microenvironment, the effect of ethanol addition during the irradiation of protein solution was studied. For the following work, looking for small particles, samples were analyzed by Dynamic Light Scattering to determine the population of different particle size in solution. Protein and protein/DEGDMA mixture were irradiated into PB solution with different amount of ethanol aliquots. For this experiment much less DEGDMA amounts were used (4 μmols) in order to avoid a detrimental solvent effect of the monomer onto the protein. Fig. 3 shown there is no measurable differences in the particle diameter of irradiated BSA protein at low ethanol concentration (less than 30%). However, when more than 30% of ethanol is used in the solution, sample reached nanoparticles.
Furthermore, irradiated protein in buffer/ethanol mixture yielded nanoparticles irrespective the presence of DEGDMA in the initial solution.

![Graph showing DLS measurements of irradiated BSA and BSA/DEGDMA solutions.]  

*FIG. 3. DLS measurements of irradiated BSA, 0.7 μmol (black bars) and BSA/DEGDMA, 0.7 μmol / 4 μmol (grey bars) dissolved in buffer with different amount of ethanol added (Total vol: 1.5 mL).*

The presence of DEGDMA reaches nanoparticles of higher sizes only when protein nanoparticles can be made. Taking into account sub-nanoscale reactive species created by gamma-rays and the nanoscale size of proteins we decide to study, in further detail, the influence of gamma-rays onto protein solutions without monomer addition.

Two initial conditions were studied; BSA dissolved in PB and a solution of PB with ethanol 40% v/v. Ethanol is a well-known protein precipitant, however more than 60 % ethanol is required to induce BSA precipitation. In this experiment, ethanol was used at sub-precipitating concentrations.

**TABLE 3. AVERAGE PARTICLE DIAMETER OF BSA SOLUTIONS MEASURED BY DLS**

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Condition</th>
<th>Diameter</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>No Irrad</td>
<td>4,8</td>
<td>1,3</td>
</tr>
<tr>
<td>BSA 40% Ethanol</td>
<td>No Irrad</td>
<td>4,8</td>
<td>1,7</td>
</tr>
<tr>
<td>BSA</td>
<td>Irrad</td>
<td>3,8</td>
<td>1,7</td>
</tr>
<tr>
<td>BSA 40% Ethanol</td>
<td>Irrad</td>
<td>20,5</td>
<td>3,5</td>
</tr>
</tbody>
</table>

In Table 3 are shown the DLS measurements of irradiated and non-irradiated protein samples. BSA irradiated in buffer shows a small diameter reduction, which can be related to degradation effect of
gamma rays [11]. However, the same protein in an aqueous/ethanol environment reached nanoparticles with dimensions higher than 20 nm after irradiation.

In a following experiment the two most common protein precipitants were studied under sub-precipitant concentration, an organic solvent and an inorganic salt. In both cases the precipitants, ethanol and ammonium sulphate, have a dewatering effect onto the solvated protein. In Fig. 4 are described the DLS measurements of irradiated protein solution at 10 kGy with addition of each modifier. The addition of increasing quantities of ammonium sulphate to the protein solution did not influence the average size of the particles after irradiation. However using ethanol, it can be found increment in the size of nanoparticles.

![FIG. 4. Particle size for BSA irradiated samples with the addition of different additives: ethanol and ammonium sulfate. Standard deviation corresponds to 3 independent samples.](image)

Other solvents were used to analyze the effect in nanoparticle formation, such as acetonitrile and isopropanol. They were used in an equivalent molar concentration to ethanol 40% v/v. As it is shown in Table 4 solvents compatible with globular proteins have similar effect than ethanol.
TABLE 4. AVERAGE PARTICLE DIAMETER OF BSA SOLUTIONS IRRADIATED AT 10 KGY WITH THE ADDITION OF THE MODIFIER. SAMPLES WERE DILUTED IN PB PREVIOUSLY TO BE MEASURED IN DLS.

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Condition</th>
<th>Diameter (nm)</th>
<th>SD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>5.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Ethanol 40%</td>
<td></td>
<td>20.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>36%</td>
<td>13.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>52%</td>
<td>34.9</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Under the experimental condition of 40% v/v ethanol, samples containing different protein concentration were irradiated. As it is shown in Fig. 8 particle diameter becomes lower with the increases of initial protein concentration. Protein concentration of 30 mg/mL was chosen for the following experimental work because shows the minimum error dispersion.

In the following experiment, the effect of irradiation dose was studied. BSA solution in buffer and buffer/ethanol 40% v/v was tested. Low dose rate (lower than 1 kGy/h) and different irradiation time was used for each condition. Light scattering measurements show than a minimum of 10 kGy was required to find out nanoparticles (Fig. 6). Protein size irradiated in buffer keep approximately their original size when it is irradiated in air. These results are in agreement with data reported of molecular weight of BSA irradiated in oxygen atmosphere [12].
Considering the study of differential properties of irradiated BSA solutions the response to dye adsorption and behavior to centrifugation and filtration was studied. Irradiated and non-irradiated BSA solutions were diluted in buffer and determined the amount of protein by Bradford assay. This assay is specific for proteins but it is based on an unspecific protein hydrophobic-dye interactions. As it is shown in Fig. 7, irradiated and non-irradiated samples showed the same amount of soluble protein. After the assay, samples was centrifugate at 14,000 rpm in a Epperdorf-type centrifuge for 30 min. Spectrophotometric determination of the dye-protein complex in supernatant shows protein depletion from the solution only in the sample corresponding to 40% ethanol, confirming the existence of nanoparticles.

In the following experiment fluorescein-labeled BSA was used to trace the BSA during the irradiation process. Fluorescence emission at 518 nm was measured after 10 kGy irradiation of
BSA with 5% of labeled BSA for the buffer/ethanol serie from 0 to 40% v/v. As it is shown in Fig. 8 the relative fluorescence of irradiated BSA against non-irradiated ones decrease proportional to the amount of ethanol in the solution. Fluorescence of BSA sample irradiated in ethanol 40% v/v has only 25% of the initial emission, which can be explained by a quenching process of aggregated BSA molecules in the nanoparticle. Irradiated samples without ethanol do not show any appreciable reduction in fluorescence emission.

![Graph showing fluorescence ratio against ethanol concentration](image)

**FIG. 8.** Fluorescence ratio at 518 nm of irradiated and non-irradiated BSA samples in buffer/ethanol mixtures. RFU means Relative Fluorescence Units.

Fluorescent labeled-BSA samples (irradiated and non-irradiated) was filtrated through 0.22 μm membrane, the usual esterilization filters. After filtration all samples show more than 95% of the initial fluorescence. This experiment verifies most of the nanogel solutions fulfill with the occurrence of particles of nanometric size.

In order to study protein structure in the nanoparticles we performed UV-vis and Circular Dicroism (CD) spectra. Near CD gives information about ternary protein structure and the simetry around aromatic aminoacids where only folded proteins have CD signal in this range. In Fig. 9 are shown the CD characterization of irradiated BSA solutions. CD spectra of the irradiated samples shows no changes in the general spectral shape, thus main conformational features of the ternary structure of the protein are preserved. However, from 20% to 40% ethanol concentration shows an increment in the CD signal. This effect could be assigned to a more rigid conformational structure of the protein.
Fig. 9. Circular dichroism of the irradiated BSA samples with different ethanol proportions.

In order to confirm a covalent chemical link between proteins, irradiated samples were diluted in Guanidinium chloride (GdmCl) 6 M, a chaotropic salt used to unfold (denaturate) proteins. In the Table 5 are determined the average particle diameter in this denaturing solution. All recorded data reached higher diameters than values plotted in Fig. 4, as can be expected for unfolded and open protein structures. The higher dispersion in data can be assigned to the random coil unstructured particles in this solvent condition. Additionally, it can be shown an inverse correlation between the initial ethanol concentration during irradiation and particle diameter in unfolded condition. Thus, it seems than higher amount of ethanol during the irradiation induce to more protein molecules keeping closer and in that way higher probability to be cross linked.

TABLE 5. AVERAGE PARTICLE DIAMETER OF BSA SOLUTIONS IRRADIATED AT 10 KGY WITH THE ADDITION OF THE ETHANOL. SAMPLES WERE DILUTED IN GDMCL 6 M 20 H PREVIOUSLY TO BE MEASURED IN DLS.

<table>
<thead>
<tr>
<th>EtOH (%)</th>
<th>Particle size</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diam (nm)</td>
<td>SD (nm)</td>
</tr>
<tr>
<td>0</td>
<td>630</td>
<td>500</td>
</tr>
<tr>
<td>10</td>
<td>650</td>
<td>200</td>
</tr>
<tr>
<td>20</td>
<td>440</td>
<td>400</td>
</tr>
<tr>
<td>30</td>
<td>190</td>
<td>140</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

Fourth derivative UV-vis spectra have been shown to be very sensitive to sense minute protein conformational changes. Alteration of the microenvironment (polarity, hydration, hidrophobic interactions, packing density) of tyrosine and tryptophan aminoacids can be follow by this
spectroscopic technique. The forth-derivative UV-vis spectra of the irradiated BSA samples were shown in Fig. 10. All curves have the same shape indicating that the microenvironment of aromatic aminoacids are keeping the same conformational features into the protein.

![Fourth-derivative UV-vis spectra of the irradiated BSA samples with different ethanol aliquots.](image)

**FIG. 10. FOURTH-DERIVATIVE UV-VIS SPECTRA OF THE IRRADIATED BSA SAMPLES WITH DIFFERENT ETHANOL ALIQUOTS.**

From CD and UV-vis data the main amount of BSA protein in the samples are keeping its native structure. Thus nanoparticles should be composed by an aggregation of protein molecules in their native-state.

Recently Akiyama et al [8] have reported the preparation of gelatin nanoparticles based on gamma irradiation of an aqueous protein solution. They also described an increase in the median particle size when a high protein concentration sample is irradiated in the range of 10 to 20 kGy. However, these results cannot be compared with ours because gelatin is not a native globular protein, thus it has not a compact and defined three-dimensional structure such as BSA.

In order to compare with a chemical crosslinking method, such as glutaraldehyde treatment, particles from BSA in BP ethanol 40% v/v have a mean diameter of 17.5 +/- 3 nm. This value is not statistically different from 20.5 +/- 3.5 nm of irradiated ones, thus seem that aggregation and crosslinking step are independent process.

When the aqueous solution involving a polymer was irradiated with gamma-rays, reactive oxygen species such as hydroxyl radicals were formed by radiolysis of water, and they eliminate the hydrogen atom from the carboxyl group or the hydroxyl group to form polymeric radicals. The three kinds of reactions such as cross-linking, main chain scission, and side chain scission occurred by these polymeric radicals.

In order to construct an hypothesis (to hypothesize an explanation) of the experimental results Radiation Target theory is used to analyze the possible source of crosslinking effect by direct hit. Considering the transfer energy that produce ionizations, the minimum energy in a primary ionization (PI) would be the ionization potential of orbital electrons. The most probable energy
transfer is ~20 eV and the average energy transfer can be considered ~60 eV. In diluted protein solutions most of the PI are in water molecules, the radiation damage is occurring via the radiolytic products of water, this is called indirect effect. It was described up to 99.9% of degradation radiation effects are via this indirect mechanism [13].

Radiation sensitivity of proteins depend on the mass of the molecule and is independent of the molecular volume or shape [14]. From the Poisson equation, the fraction of non-damage molecules \( F \) after a radiation dose, \( D \), can be calculated as:

\[
F = \frac{N}{N_0} = e^{-qmD}
\]  

Where \( N \) and \( N_0 \) is the number of non-damage and initial molecules, respectively. The \( m \) is the mass an \( q \) include the average energy deposition (65 eV) and conversion factors [13]. From the Eq.(1) and considering 10 kGy as the minimum dose to reach nanogels, and 66 kDa of BSA molecular weight, the \( F \) of BSA is equal to 0.90. Thus only 10% of the BSA molecules has the probability to have at least one direct PI. The energy deposited in that interaction ultimately results in breakage of chemical bonds randomly throughout an entire polypeptide. Considering than a single molecule has a 2.4 nm radii and nanoparticle around 10 nm, a rough estimation gives around seventy close packed molecules to reach a nanoparticle. Taking into account the direct PI onto the macromolecule previously described, only around ten crosslinked bonds are created per nanoparticle. Thus direct hit should be not enogh to explain the nanoparticle stabilization and some indirect effect should be considered.

4. CONCLUSION

Radiation effect onto the proteins, such as fragmentation and aggregation process, has been studied by many authors, as well as modifications of primary, secondary and ternary structure of BSA [15,11]. However proteins have not been used as building blocks combined with radiation technology.

In this work, for the first time, a soluble and globular protein such as BSA was used as basic unit to build nanoparticles without the addition of any additional chemical crosslinker. Detectable protein nanoparticles were reached by irradiation of protein solution in the presence of polar organic solvents. By changing the environmental condition, such as the solvent composition, aggregation clusters could be dynamically created in solution, which are radiation crosslinked. From theoretical data direct hit onto proteins should be not enough to explain the nanoparticle formation. Spectroscopic data showed protein molecules keep its general three-dimensional structure into the created nanoparticles.

Acknowledgements

The authors thank to CONICET, MINCyT and IAEA for grants.
REFERENCES

ENCAPSULATION AND NANO-ENCAPSULATION OF PAPAIN ACTIVE SITES TO ENHANCE RADIOLITYC STABILITY AND DECREASE TOXICITY

A.B. Lugão, G.H.C. Varca, M.B. Mathor, P. Santos Lopes, M.S.S. Rogero, J.R. Rogero; BRAZIL

Summary
Papain is used as an ingredient in various enzymatic debridement preparations. Those paste-like preparations are based on water solution and usually are sterilized by radiation. As a consequence, there is a major decrease in papain activity. Papain containing preparations are used in chronic wounds treatment in order to clean and remove the necrotic tissue. However FDA (2008) is taking an action against such products due to severe adverse events reported in patients submitted to papain treatments. Thus, the main goal of this proposal is to develop encapsulated papain containing membranes based on hydrogels and silicone rubber in an attempt to achieve a controllable distribution of size and delivery profile, a toxicity reduction and provide stability towards radiation processing through molecular encapsulation with β-cyclodextrin, which may also provide protection to the enzyme against radiation induced radiolysis.

1. INTRODUCTION

The use of medicinal products containing enzymes has increased due to their broad therapeutic potential. Currently such products are being used in the pharmaceutical and medicinal field for several applications, such as debridement and absorption enhancer agents. Papain (Carica papaya Linné) is a proteolytic enzyme isolated from the papaya latex, green fruit and leaves (SANGEETHA & ABRAHAM, 2006) which has been extensively used in wound healing and scars treatment.

Enzymes in general, such as papain, require specific environments in order to maintain their bioactivity. Due to its molecular complexity this enzyme in particular may be easily inactivated by denaturation processes induced by high temperatures, the presence of denaturant agents as well as inorganic agents among other substances (POLIZZI et al., 2007).

Papain in particular, is recognized in eschars and wound treatments, where a wide area has been affected and a quick debridement is required (ALVAREZ et al., 2002), and is commercially available in medicines and cosmetic products for peeling purposes.

Recently, the Department of Health and Human Services of the Food and Drug Administration (FDA) imposed restrictions to papain applications once several adverse effects related to topical papain products were reported raising serious safety concerns regarding these products. and now such products require an approval of this organization prior to their commercialization (FDA, 2008).

Such restrictions raised the importance of finding alternatives to solve these concerns and obtain an adequate product or reduce the undesirable reactions of the commercially available papain containing formulations. Among other biophysical methods, radiation may not only represent an effective sterilizing method but an alternative to enhance biomolecules intrinsic characteristics and reduce their toxicity, once previous studies pointed out the irradiation technology ability in reducing the allergenicity of certain foods (BYUN et al., 2002).
Ionizing radiation is known as the best method to destruct pathogens and deteriorating microorganisms (WHO, 1999). Gamma radiation is capable of sterilizing a product once the substance is exposed to gamma rays, which induce the covalent bonds cleavage due to a direct impact over the molecules and after reaching the water molecules, it is also capable of disrupting them leading to a free radicals release which may be harmful to other molecules (EAGLE, et al., 2005). Ionizing radiation has also been proposed to control the clusters size, which may reduce the aggregates formation, leading to an enhanced enzymatic activity and also conferring a longer stability.

**General Goals**
- To develop membranes based on nanostructured hydrogels to achieve a major reduction in toxicity.
- Establish the methodology for assessing papain activity

**Specific Goals**
- To establish the methodology for assessing papain activity under different radiation doses;
- To synthesize PVP hydrogels, at different polymer concentrations, with/without agar.
- Evaluate the synthesized hydrogels in terms of mechanical properties, swelling and gel fraction

**Expected Outputs**
- Development of methodologies to prepare and characterize nanogels based on papain complexed with CD;
- Publication of the results under the IAEA Radiation technology series.

2. EXPERIMENTAL PART

**Methods**

**Enzymatic assay**

The prepared solutions were transferred to a 96 wells microplate and added to each corresponded well in the following order: substrate, papain (respective dilution) and acetic acid 30% (v/v) to stop the reaction. However at the beginning of the experiment (time 0), the acetic acid was added to prior to papain addition to avoid the reaction to take place.

During the experiment preparation all solutions were kept on ice in order to control the beginning of the reaction, and covered with aluminum foil to avoid light exposure. The experiment was kept in a MARCONI® Model MA 159BB water bath at 40°C for 45’ minutes. The reaction was interrupted at every 15 minutes with acetic acid starting at time 0 and ending at 45 minutes. The UV analyses were performed and a linear curve was constructed to determine the increase in absorbance depending on the concentration of papain over time.

**Microplate preparation**

The wells were filled with 120μL substrate solution, 100 μL papain dilution, 50μL acetic acid 30% v/v in the wells of the subsequent lines every 15 minutes in order to stop the reaction.
Validation of the analytical methodology
Validation was performed following the parameters of US Pharmacopeia. The analytical characteristics analyzed were accuracy, precision, limit of quantification, limit of detection and linearity.

Linearity
Linearity was determined by analysis of seven different papain concentrations in order to estimate the correlation coefficient \( r = 0.99 \). The correlation coefficient is calculated by the analytical curve or calibration curve.

Precision
Precision is expressed as relative standard deviation or coefficient of variation (CV\%) by the EQ 1:
\[
\text{RSD} = \frac{\text{SD}}{\text{CMD}} \times 100
\]
RSD = relative standard deviation, DP = standard deviation, CMD = average concentration determined (average of 10 determinations)

The precision evaluation was performed through the coefficient of variation calculated according to the equation bellow based on ten absorbance determinations at 1777USP.ml-1 papain concentration.

Accuracy
The accuracy can be expressed by the EQ 2:
\[
\text{A} = \frac{\text{EMC}}{\text{TC}} \times 100
\]
A = Accuracy, EMC = experimental mean concentration, TC = theoretical concentration
A recovery test was established using a known enzyme concentration of 2666,6USP.ml-1 and the amount of enzyme solution was quantified by the enzymatic assay (ERLANGER et al., 1961).

Limit of detection
The limit of detection (LOD) was established by the construction of 3 calibration curves with papain concentrations 444, 888, 1777, 2666, 3555, 4444 and 5333,3 USP.ml-1 and is expressed by the EQ 3:
\[
\text{LOD} = \frac{\text{SD}_{\text{a}}}{3} \times \text{IC}
\]
SDa = standard deviation of the intercept with y axis of the 3 calibration curves IC = slope of calibration curve

Limit of quantification
The limit of quantification (LOQ) was established by analyzing decreasing concentrations of papain. The respective concentrations were 444, 888, 1777, 2666, 3555, 4444, 5333,3USP.ml-1. Three calibration curves were built, and the results were applied according to the EQ 4:
\[
\text{LOQ} = \frac{\text{SD}_{\text{a}}}{10} \times \text{IC}
\]
SDa = standard deviation of the intercept with y axis of the 3 calibration curves, IC = slope of calibration curve

Hydrogel – Formulation studies
The hydrogels were prepared by mixing PVP, PEG, agar, and distilled water. The formulations were heated to provide complete dissolution of the components. Then, the solutions were put into plastic moulds, sealed and irradiated with \( \gamma \) radiation from a \( ^{60} \text{Co} \) source. The dosage used applied
was 25 kGy in all cases at a dosage rate of 1.98kGy/h. The hydrogels were allowed to stabilize for 7 days prior to the beginning of the experiments.

Characterization

Gel fraction

The concentration of the cross-linked material, forming the insoluble fraction, was estimated based on the ASTM D 2765-01 with some modifications. The Hydrogels were packaged in stainless steel 500 mesh porous bag. After dried in stove at 60°C until a constant weight was reached, the sample was submitted to extraction using a soxhlet and distilled water as solvent. After 40h the bags were dried and re-weighed until constant weight was reached. The gel fraction was calculated as Eq.(1), using the initial weight of the dry gel (Wi) and the weight of the extracted dry gel (Wd). The gel fraction estimated corresponded to the average of data of 3 analyzed specimens of each formulation.

\[
\text{Gel(\%)} = \frac{W_d}{W_i} \times 100
\]  

(1)

Swelling

Reverse Osmosis Water was used for the investigation of swelling properties of the hydrogels at room temperature. At a given time, each specimen was removed from the water to be sieved where it was carefully wiped by filter papers and then weighted. This procedure was repeated several times up to 48h. The swelling percentage of hydrogels was calculated based on Eq. (2), which consists of the difference between the initial and the final weight of the sample divided by the initial weight. Where: Ws corresponds to the weight of the swollen gels and Wd to the gel weight before immersion. Tree samples of each formulation were analyzed in order to estimate the swelling capacity of the hydrogel.

\[
\text{Swelling(\%)} = \frac{W_s - W_d}{W_d} \times 100
\]  

(2)

Mechanical properties

A Stable Micro System, model TA.XT plus, texture analyzer equipped with a 50Kg load cell was used to measure the strain and stretched of the hydrogels. The tensile strength of hydrogels were evaluated according to the ASTM DD882, however the stress and strain (\%) at break of hydrogels were measured using rectangular specimens measuring of 24 x 100mm.

The stress was calculated based on the cross-section area of each sample and the strain based on its relative elongation in percentage from its original length (lo = 60mm). The matrices were stretched at a strain rate of 8.33mm.s –1 for the samples A, C and D. Sample B was stretched at a rate of 4.16mm.s –1, as recommended by the currents standards. The pre-test speed adopted was 2.0mm.s–1 and the trigger force was set to 5g.
SEM Analysis
The hydrogel Samples were immersed in liquid nitrogen and submitted to lyophilization in 24 hours cycles. Gold Sputtering technique was applied to provide adequate contrast for the sample. The samples were fractured and analyzed using a Scanning Electron Microscopy.

Preliminary Results

On our previous report, we aimed to clarify and understand the influence of the radiation over papain activity in typical cosmetic and medicinal formulations, regarding different media, such as powder, aqueous and buffer solution, and gel-like formulation. We also evaluated the stability of the enzyme in each media. The main purpose of this part of the work was to contribute to a better understanding of the caused biological changes in the enzyme due to the radiation exposition.

The results revealed that the viability of the gamma radiation process was related to the medium, its components and the radiation dose, pointing out that the radiation technique may be applied to papain, even though biological changes, including conformational and activity variations, may occur. Thus, process monitoring and careful planning are essential to assure the effectiveness of the methodology.

Determination of papain Activity
Our next step corresponded to meet another specific goal, essential to further steps involved in the research, the establishment of the methodology for assessing papain activity; through literature analysis, a colorimetric method to quantify papain activity was selected and performed validation studies to assure the efficacy and applicability of the method. This part was performed with the collaboration of the undergraduate student, Ms. Caroline C. Ferraz, from the University of Sorocaba – UNISO, Brazil.

The assessment of the enzymatic stability is fundamental to any study, research or industrial process involving this particular group of protein macromolecules (RAJ LAD, 2006), once it provides data about the bioactivity of these molecules, which also indicates the viability or interference of a particular process or reagent.

The current techniques available in literature that assay papain activity are in a short number and are often linked to intensive laboratory work, take long periods of time for the analysis, and involve high cost equipment, reagents and substrates (PINTO, et al., 2007).

Previous researches have evaluated the kinetics of the papain catalyzed hydrolysis over N-Benzoyl-DL-arginine-P-nitroanilide and highlighted the potential application of such substrate in papain activity assays (MOLE & HORTON, 1973). This particular compound is a low molecular weight substrate that releases P-nitroaniline as product after contact with active papain. This substrate is cheap and the product formation is evaluated using UV techniques.

However, validation studies must be performed in order to assure the ability of any analytical method to generate reliable and interpretable information, as well as confirm that the features implemented in the method meet the standards for the analytic applications applied (GÜNZLER, 1996), through consistently documented evidence, established by laboratory studies.
Thus, the validation of the technique to assay papain activity using the N-Benzoyl-DL-arginine-P-nitroanilide as a substrate was carried out in order to guarantee its accordance with the technical specifications as well as assure the applicability of the selected methodology and the validity of the results.

The enzyme activity was measured based on the enzyme ability to cleave an amide bond in a low molecular weight substrate N-Benzoyl-DL-arginine-P-nitroanilide. The P-nitroaniline formed during the reaction of hydrolysis of the product substrate can be followed colorimetrically using ELISA reader. Thus, the amount of substrate hydrolyzed can be calculated according to the absorbance of P-nitroaniline at 405nm depending on reaction time (ERLANGER, 1961).

From the results obtained by the reaction of the enzyme with the substrate as a function of time the curve calibration was constructed and is shown in Fig. 1

A proportional relation between papain concentration and increased rate of formation of the compound colorimetric was observed (Figure 1). The linear correlation coefficient was 0.9978 which is consistent with the limit presented in the literature [X] (R2 = 0.99) demonstrating linearity of the analytical method.

The precision value was equal to 1.77%. The coefficient of variation is obtained due to low concentrations of papain used in this method, showing that the result is in accordance with the specifications. The results of the recovery test revealed an accuracy of 94.15%, while the limits of detection and quantification corresponded to 123.9825 USP/mL-1 and 413.2785 USP/mL-1 respectively.

![FIG. 1. Calibration curve.](image)

Hydrogel

In this report we are presenting the results of the preliminary synthesis of PVP hydrogels with different formulations, using gamma radiation, in order to screen formulation possibilities for papain delivery, considering that the hydrogel drug release relies upon the degree of crosslinking (KEITA et al., 1990) and the structure of the polymeric net. Agar was added to some of the formulations in order to produce more adequate hydrogels. To achieve these goals eighteen
formulations were synthesized and characterized through the determination of the swelling capacity, gel fraction, and mechanical properties.

The formulations and the main results obtained are presented in the Table 1. It’s relevant to note that such part was developed under collaboration with Ms. Diana Rodriguez-Linares, Fellowship code No.: CUB/09034, from CUBA.

### TABLE 1. ASSAYED FORMULATIONS AND RESPECTIVE RESULTS FOR THE PVP HYDROGELS SYNTHESIZED

<table>
<thead>
<tr>
<th>Formulations</th>
<th>% PVP</th>
<th>% PEG</th>
<th>% Agar</th>
<th>% Swelling</th>
<th>Gel fraction</th>
<th>Mechanical properties</th>
<th>Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Traction/Elastic module (MPa)</td>
<td>Compression. F (N)</td>
</tr>
<tr>
<td>F-1*</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>95.39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F-2*</td>
<td>6</td>
<td>1.5</td>
<td>0</td>
<td>121</td>
<td>76.62</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F-3*</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>263</td>
<td>44.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F-1B</td>
<td>6</td>
<td>0</td>
<td>0.5</td>
<td>27</td>
<td>90.02</td>
<td>0.00025</td>
<td>45.22</td>
</tr>
<tr>
<td>F-2B</td>
<td>6</td>
<td>1.5</td>
<td>0.5</td>
<td>127</td>
<td>64.54</td>
<td>0.000025</td>
<td>53.56</td>
</tr>
<tr>
<td>F-3B</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>201</td>
<td>44.17</td>
<td>0.00029</td>
<td>27.78</td>
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<tr>
<td>F-1C</td>
<td>6</td>
<td>0</td>
<td>1.5</td>
<td>49</td>
<td>78.23</td>
<td>0.00082</td>
<td>50.57</td>
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<tr>
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<td>1.5</td>
<td>1.5</td>
<td>104</td>
<td>46.47</td>
<td>0.00080</td>
<td>40.87</td>
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<tr>
<td>F-3C</td>
<td>6</td>
<td>5</td>
<td>1.5</td>
<td>92</td>
<td>41.43</td>
<td>0.00087</td>
<td>30.41</td>
</tr>
<tr>
<td>F-1D</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>242</td>
<td>90.54</td>
<td>0.00017</td>
<td>93.33</td>
</tr>
<tr>
<td>F-2D</td>
<td>15</td>
<td>1.5</td>
<td>0</td>
<td>438</td>
<td>82.15</td>
<td>0.000010</td>
<td>55.35</td>
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<tr>
<td>F-3D</td>
<td>15</td>
<td>5</td>
<td>0</td>
<td>504</td>
<td>67.73</td>
<td>0.000004</td>
<td>62.40</td>
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<tr>
<td>F-1E</td>
<td>15</td>
<td>0</td>
<td>0.5</td>
<td>218</td>
<td>93.42</td>
<td>0.000044</td>
<td>110.34</td>
</tr>
<tr>
<td>F-2E</td>
<td>15</td>
<td>1.5</td>
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<td>256</td>
<td>85.70</td>
<td>0.00043</td>
<td>41.81</td>
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<td>F-3E</td>
<td>15</td>
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<td>0.5</td>
<td>282</td>
<td>92.97</td>
<td>0.00062</td>
<td>73.65</td>
</tr>
<tr>
<td>F-1F</td>
<td>15</td>
<td>0</td>
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<td>185</td>
<td>87.12</td>
<td>0.00119</td>
<td>73.93</td>
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<tr>
<td>F-2F</td>
<td>15</td>
<td>1.5</td>
<td>1.5</td>
<td>124</td>
<td>72.57</td>
<td>0.00141</td>
<td>105.58</td>
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<tr>
<td>F-3F</td>
<td>15</td>
<td>5</td>
<td>1.5</td>
<td>121</td>
<td>68.58</td>
<td>0.00168</td>
<td>108.13</td>
</tr>
</tbody>
</table>

Scanning Electron Microscopy (SEM)
Following we are presenting the pictures taken by SEM at different resolution for the selected samples (F-2B, F-2C, F-2D, F-2E, F-2F, F-3E, and F-3E-P). The selection of the samples for this analysis is an attempt to understand the effects of PEG, agar, and the effect of papain inclusion on the PVP membranes formulation and consequently on the final structure. However, the results are still too preliminary to allow a proper conclusion of the addition of such substances.
It is well known that agar induces more reticulation as well as a more polycrystalline structure on the PVP membranes, fact also observed in our experiment, where F-2C formulation contained 6% PVP and 0.5% agar, while F-2D contained 15% PVP, and both membranes presented an adequate hydrogel formation.
The microscopies also indicated that (figure 3) the effect of PEG percentage over the membrane formation was observed in terms of increasing the homogeneity of the hydrogel. Moreover, comparing F-3E pictures with the pictures of the hydrogel with same formulation including papain at 2% (F-3E-P, Figure 4), it could be observed how the presence of papain leads to a membrane with major porosity, and a wide variety of pore sizes, probably due to the agglomeration of the protein molecules.

Considering all the factors previously described, five formulations were selected for the next stage (Table 3) of the present research.
TABLE 3. SELECTED PVP HYDROGELS FORMULATIONS

<table>
<thead>
<tr>
<th>Formulations</th>
<th>% PVP</th>
<th>% PEG</th>
<th>% Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 F-2B</td>
<td>6</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2 F-2C</td>
<td>6</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>3 F-2D</td>
<td>15</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>4 F-2E</td>
<td>15</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>5 F-2F</td>
<td>15</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Even though differences were observed for the membranes, the results are still too preliminary and thus, do not allow proper conclusion. However, relevant differences were observed among the assayed membranes considering the homogeneity and crosslinked polymeric structure. Even though some hydrogels were selected, an evaluation of the papain stability inside the membranes is still required in order to allow a proper selection.

On the other hand, the methodology validated for the enzyme activity quantification was suitable for the purpose. The linearity of absorbance versus reaction time was satisfactory and the ratio of hydrolysis was proportional to the concentration of enzyme: between 40-480 mg/mL-1. The chromogenic substrate used to measure the papain activity was sensitive to the method.

**Work plan for next meeting**

- **To promote molecular encapsulation of the papain amino acid residues using β-cyclodextrin:**

  By means of evaluating the stability of such complex, establishment of the influence of the radiation over the complex stability and the radioprotective effect conferred, as well as the determination of the overall effects of the complex formation over the allergenic and cytotoxic properties of the enzyme. These data will be obtained through spectrophotometry analysis, cytotoxicity assay – IC10 and IC50, Antigen-antibody reaction - Ouchterlony double immunodiffusion technique, Activity assays – BAPA, and Kinetics measurements - Km and Vmax;

- **Develop a nanostructured hydrogel for papain/CD complex delivery:**

  In terms of synthesis, development and control of the hydrogel nanostructure – by crosslinking density measurements – through the determination of Tg – DSC or/and DMA, modulus – swelling and gel fraction, to promote adequate release of the papain/CD complex. These data will be estimated using the Flory-Rehner Equation. The stability, citotoxicity and enzymatic activity will be determined by the established techniques and the release will be determined by permeation studies using Frans static diffusion cells apparatus.
Collaborations
See attached table.

REFERENCES

RADIOLYTIC SYNTHESES OF NANOPARTICLES AND INORGANIC-POLYMER HYBRID MICROGELS

Q. Chen, J. Shi, R. Zhao, X. Shen, CHINA, PEOPLE'S REPUBLIC OF

Summary
In the second year of the project, we have gotten progress mainly in two directions. Firstly, for the first time, Prussian blue (PB) nanoparticles (NPs) were successfully synthesized by the partly radiolytic reduction of Fe$^{3+}$ and Fe(CN)$_6^{3-}$ in the presence of poly(N-vinyl pyrrolidine) (PVP) under N$_2$ atmospheres at room temperature. With the increase of the concentration of PVP, the size and the size distribution of the synthesized quasi-spherical PB NPs decreased obviously, leading to a hypsochromic shift on their peak position of the characteristic absorption. In the experiment, we further found that the smaller ones have a larger capacity to Cs$,+$, suggesting that the application of PB NPs in curing thallotoxicosis may decrease the usage of PB for the patient to great extent. Secondly, through a series of preliminary experiments, we got a clear picture about the one-step radiolytic preparation of inorganic-poly(methacrylic acid-co-methyl methacrylate) hybrid microgels by surfactant-free emulsion polymerization. Besides, unpurified N-carbamothioylmethacrylamide was synthesized via the methacrylation of thiourea. These created favorable conditions for the one-step synthesis of metal sulfide-poly(methacrylic acid-co-methyl methacrylate) hybrid microgels by $\gamma$-irradiation and surfactant-free emulsion polymerization.

1. RADIOLYTIC SYNTHESES OF INORGANIC NANOPARTICLES AND NANOSTRUCTURES IN AQUEOUS SOLUTIONS

1.1. Introduction
In the realm of nanoscience and nanotechnology, the largest activity has been focused on the synthesis and application of new nanoparticles (NPs) with different sizes and shapes [1-5]. Recently, the synthesis of NPs for ion-exchange began to attract much attention [6-9]. Similar to other properties, the ion-exchange performance of NPs is also affected by their morphologies. So far, the present results indicated that only a few special morphologies of some NPs (such as nanofibers, porous and layered structures) have higher exchange capacity to some heavy metal (i.e., Hg$^{2+}$, Pb$^{2+}$), alkali metal and alkaline earth metal (i.e., Cs$^+$, Sr$^{2+}$, Ba$^{2+}$) ions. However, to the best of our knowledge, there is no report about the size effect of NPs on the exchange performance. It is believed that the systematic exploration on the NPs for ion-exchange will benefit their application in industry as well as in the field of biomedicine.

Among the numerous methods of preparing NPs, ionizing radiation (such as $\gamma$-irradiation, electron beam irradiation and so on) is powerful, since it can conveniently produce a series of species with tunable redox potentials, not be achievable by other means, in a wide range of temperature [10-13]. By far, besides few report about metal halide and nonmetal NPs [14-17], great efforts have been focused on the syntheses of metal, core-shell metal or alloy, and metal chalcogenide NPs in aqueous solution and organic solvent [10-13,18-19]. In the past several years, we tried our best not only to extend the application realm of ionizing radiation in the preparation of NPs, but also to explore the possible usage of the obtained NPs in the field of biomedicine.
1.2. Syntheses of Prussian blue nanoparticles for cesium ion-exchange

As is well known, ferric ferrocyanide, i.e., Prussian blue (PB), is not only an old blue dye, but also an efficient toxicide for thallotoxicosis. If the nano-sized PB particles have larger exchange capacity and faster exchange rate, the usage of PB for the patient will decrease to great extent. Nevertheless, the similar issue did not appear in the literature. This year, for the first time, the radiolytic synthesis of PB NPs was realized by us. Then, we paid more attention to the above-mentioned subject.

Under N\textsubscript{2} atmosphere, part of Fe\textsuperscript{3+} and Fe(CN)\textsubscript{6}\textsuperscript{3-} ions in the aqueous solution (pH = 2.8) were simultaneously reduced to Fe\textsuperscript{2+} and Fe(CN)\textsubscript{6}\textsuperscript{4-} ions by the radiolytically generated hydrated electrons (e\textsubscript{aq}\textsuperscript{-}) and H atoms (Eqs. 1-6).

\[
\begin{align*}
H_2O \xrightarrow{\text{irradiated}} & e_{aq}^{-}, H, \cdot OH, H_3O^{+}, \ldots \quad (1) \\
H_3O^{+} + e_{aq}^{-} & \longrightarrow H \quad k = 6 \times 10^{10} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \quad (2) \\
Fe^{3+} + e_{aq}^{-} & \longrightarrow Fe^{2+} \quad k = 6 \times 10^{10} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \quad (3) \\
Fe(CN)_{6}^{3-} + e_{aq}^{-} & \longrightarrow Fe(CN)_{6}^{4-} \quad k = 3.1 \times 10^{9} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \quad (4) \\
Fe^{3+} + H & \longrightarrow Fe^{2+} + H_3O^{+} \quad k = 5 \times 10^{7} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \quad (5) \\
Fe(CN)_{6}^{3-} + H & \longrightarrow Fe(CN)_{6}^{4-} + H_3O^{+} \quad k = 3.9 \times 10^{9} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \quad (6)
\end{align*}
\]

Then, PB NPs were generated by precipitating the obtained Fe(CN)\textsubscript{6}\textsuperscript{4-} ions with the residual Fe\textsuperscript{3+} ions. With respect to the Fe\textsuperscript{2+} ions, they were precipitated by the residual Fe(CN)\textsubscript{6}\textsuperscript{3-} ions, leading to the formation of thenard's blue NPs. Because PB and thenard's blue have the same structure, pure PB NPs were successfully obtained, which was confirmed by their X-ray diffraction pattern and X-ray photoelectron spectrum.

To obtain monodispersed PB NPs, several kinds of protective agents were tested. Meanwhile, poly(N-vinyl pyrrolidine) (PVP) was the most effective. The added PVP can eliminate the radiolytically generated oxidative ·OH radicals and produce a reductive circumstance, favoring the generation of PB NPs. Besides, PVP can effectively protect the generated PB NPs from aggregation and growth. With the increase of the concentration of PVP, the size and the size distribution of the synthesized PB NPs decreased obviously (Figure 1 and Table 1).
FIG. 1. TEM images of the samples synthesized at the different concentration of PVP (from A to F, [PVP] = 11.1, 22.2, 33.3, 55.6, 77.8, 77.8 mg L⁻¹). The irradiation time and the dose rate are fixed at 1000 min and 10 Gy·min⁻¹, respectively.

As an old blue dye, the color of PB comes from the electron transfer between Fe (II) and Fe (III) in PB, i.e., FeIII(t₂g 3 e₈ 3)–CN–FeII(t₂g 6) and FeII(t₂g 4 e₈ 2)–CN–FeIII(t₂g 5). As to their UV-vis spectra, the peak position of the characteristic absorption is always larger than 700 nm [20-22]. However, as far as we know, there is no report about the size effect on the optical properties of PB NPs. Here, with the decrease of the size and the size distribution of the synthesized PB NPs, their peak position of the characteristic absorption has a hypsochromic shift from larger than 900 nm to 735 nm, which presents an obvious size effect.


<table>
<thead>
<tr>
<th>[PVP] mg L⁻¹</th>
<th>11.1</th>
<th>22.2</th>
<th>33.3</th>
<th>44.4</th>
<th>55.6</th>
<th>66.7</th>
<th>77.8</th>
<th>88.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>φ/μm</td>
<td>50~200</td>
<td>80~200</td>
<td>80~170</td>
<td>80~150</td>
<td>80~120</td>
<td>70~100</td>
<td>70~100</td>
<td>~70</td>
</tr>
<tr>
<td>λmax/μm</td>
<td>&gt;900</td>
<td>880</td>
<td>830</td>
<td>805</td>
<td>795</td>
<td>763</td>
<td>750</td>
<td>735</td>
</tr>
<tr>
<td>Q/mmol·g⁻¹</td>
<td>0.285</td>
<td>0.299</td>
<td>0.389</td>
<td>0.426</td>
<td>0.426</td>
<td>0.389</td>
<td>0.426</td>
<td>0.426</td>
</tr>
</tbody>
</table>

Because of the hypotoxicity of Tl⁺ and the similar properties between Cs⁺ and Tl⁺, in our experiment, Cs⁺ was used as a simulacrum of Tl⁺ to investigate the ion-exchange performance of the obtained PB NPs. It was found that they have a quicker exchange rate. In detail, the
equilibration time is about 60 min. With the decrease of the size and the size distribution of the synthesized PB NPs, their exchange capacity has a remarkable increase from 0.285 mmol·g⁻¹ to 0.426 mmol·g⁻¹ (Table 1), which also presents an obvious size effect. Moreover, the ion-exchange between Cs⁺ and PB NPs is in accordance with the isothermal equation of Freundlich. This may be ascribed to the larger surface area of the smaller PB NPs and the exchange mainly takes place on the surface of PB NPs. Therefore, the application of PB NPs in curing thallotoxicosis may decrease the usage of PB for the patient to great extent.

This part of work has been prepared as a manuscript, which will be submitted as soon as possible.

1.3. Radiolytic syntheses of mesoporous BaSO₄ microspheres

Method 1

In our previous work, “solid” BaSO₄ microspheres, mainly consisting of quasi-spherical NPs, have been synthesized by precipitating Ba²⁺ ions with SO₄²⁻ ions, which were generated from the reduction of K₂S₂O₈ in the presence of EDTA at an absorbed dose of 6 kGy under N₂ atmosphere by γ-irradiation [23]. According to the results of N₂ adsorption-desorption isotherm experiment, the “solid” BaSO₄ microspheres synthesized at an absorbed dose of 5 kGy under N₂ atmosphere are mesoporous and the diameter of the pores is about 4 nm, which is not easy to be found by SEM (Figures 2A and 2B). While the absorbed dose increases to 20 kGy, most of the synthesized BaSO₄ microspheres have an obvious mesoporous structure (Figure 2C) and the pore size is in the range of 20–60 nm (Figure 2D). This is the first report about the synthesis of mesoporous BaSO₄ microspheres with a larger pore size. The results of fragment analysis show that the former is constructed by quasi-spherical NPs and few irregular nanorods (Figure 3D), while the latter mainly consist of irregular nanorods and there is a small angle between the building blocks, leading to the radial arrangement of the nanorods (Figure 3A-3C). The generation of BaSO₄ has been confirmed by XRD, XPS and SAED (selected area electron diffraction) analysis.

FIG. 2. SEM images of the samples synthesized with different absorbed doses: (A and B) 5 kGy, (C and D) 20 kGy.
It is confirmed that the mesoporous microspheres with a larger pore size evolved from the mesoporous microspheres with a smaller pore size, which are generated at the early stage of irradiation course and are mainly constructed by quasi-spherical NPs, through Ostwald ripening (Scheme 1). In the process, some species may preferentially adsorb on the surfaces parallel to the [001] axes of BaSO$_4$ NPs, resulting in the formation of nanorods. It is the radial arrangement and the irregular shape that cause the generation of the mesoporous structure with larger pore size. Moreover, the controlled release of SO$_4^{2-}$ and thus the slow generation rate of BaSO$_4$ may result in the in-situ formation of monolayer of BaSO$_4$ microspheres on the glass, which is of great importance for the application of BaSO$_4$ particles.

**Scheme 1.** Growth mechanism of mesoporous BaSO$_4$ microspheres with a larger pore size.
This is the work finished in the first year. This year, part of them has been published in *Crystal Growth & Design* as an communication. As to the specific species leading to the morphology transformation of BaSO₄ NPs, the preliminary experiment suggest that this may be the result of the coactions of S₂O₈²⁻ and H₂O⁺. The detail mechanism is still under investigation.

**Method 2**

Under N₂O atmosphere, most of the synthesized BaSO₄ microspheres have an obvious mesoporous structure (Figure 4A) and the pore size is in the range of 20–60 nm (Figure 4B). The mesoporous BaSO₄ microspheres with a larger pore size mainly consist of irregular nanorods, too.

![FIG. 4. SEM images of the BaSO₄ microspheres synthesized under N₂O atmospheres by γ-irradiation at an absorbed dose of 6 kGy.](image)

Possible mechanism: eₐq⁻, one of the radiolytic product of water, is transformed to ·OH (Eq. 7) under N₂O atmosphere, which then is eliminated by EDTA, leading to the generation of EDTA radical (Eq. 8); it may be

\[
e_{aq}^- + N_2O \rightarrow \cdot OH + OH^- + N_2 \tag{7}
\]

\[
\cdot OH + EDTA \rightarrow EDTA\cdot + H_2O \tag{8}
\]

\[
EDTA\cdot + S_2O_8^{2-} \rightarrow SO_4^{2-} \tag{9}
\]

\[
S_2O_8^{2-} + e_{aq}^- \rightarrow SO_4^{2-} + SO_4^+ \cdot \tag{10}
\]

the EDTA radicals that play a key role in the reduction of S₂O₈²⁻ (Eq. 9). While under N₂ atmosphere, eₐq⁻ is the main reductive species (Eq. 10). Because the reduction potential of eₐq⁻ is −2.9 V, much lower than that of EDTA radicals, the reduction rate of S₂O₈²⁻ under N₂ atmosphere is much faster than that under N₂O atmosphere. The slower reduction rate favors the formation of nanorod. This is the work finished in the first year, too.

As the above-mentioned description, Ostwald ripening play an important role in the formation of mesoporous BaSO₄ microspheres with a larger pore size. To eliminate the disturbance from Ostwald ripening, a contrast experiment at a higher dose rate and within a shorter time is necessary. However, for the lower dose rate of the ⁶⁰Co source in our institute, the related work is delayed, which will be altered at the end of this year.
Applications

Because BaSO₄ is inert in many chemical reactions and a large pore size favors the mass exchange, the mesoporous BaSO₄ microspheres with a larger pore size may act as promising candidates for catalyst carrier, adsorbent and so on. In the plan of this year, we are going to explore the immobilization of horseradish peroxidase in the mesoporous BaSO₄ microspheres with different pore size and the following catalysis effect on the reaction between H₂O₂ and o-phenylenediamine. Nevertheless, in our experiment, the immobilization capacity of horseradish peroxidase in the mesoporous BaSO₄ microspheres is too small to catalyze the planned reaction. Therefore, we will turn to explore the application of the mesoporous BaSO₄ microspheres in the field of ion-exchange in the next year.

1.4. Controllable radiolytic reduction of Cu²⁺ by β-cyclodextrin

Because ionizing radiation can decompose cyclodextrins (CDs), the safe absorbed dose was determined to be 10 kGy at the dose rate of 41.9 Gy/min by using pyrene as a fluorescent probe. Within the safe absorbed dose, the increase in the concentration of β-CD could transform the reduction product of Cu(NO₃)₂ from Cu₂O to Cu. When the concentration of β-CD increased to 8.0 mmol/L, the reduction product of Cu(NO₃)₂ became pure Cu NPs. During the irradiation course, β-CD is able to scavenge hydroxyl radical, which favors the generation of Cu. Moreover, the Cu NPs may be stabilized in a hydrophobic circumstance, such as the core of the micellar-type CD aggregates.

When the concentration of β-CD was 8.0 mmol/L, the reduction product of CuSO₄ was Cu₂O. This may be ascribed to the reason that NO₃⁻ can form inclusion complex with CD, while SO₄²⁻ cannot.

This is the work finished in the first year, which was published in this year in Acta Physico-Chimica Sinica as an invited research article for the Centennial Celebration of Chemical Research and Education at Peking University. In this year, we planned to radiolytically synthesize Ag NPs protected by β-CD for antimicrobial test. Unfortunately, only black Ag₂O NPs were obtained. As for the mechanism and the solution scheme, it is still in studying.

2. PREPARATION OF INORGANIC-POLYMER HYBRID MICROGELS BY γ-IRRADIATION AND SURFACTANT-FREE EMULSION POLYMERIZATION

2.1. Introduction

As a member of inorganic-polymer nanocomposite microspheres, hybrid microgels have been used extensively in the fields of coatings, colloid crystals, catalysis, diagnostics and so forth [24-27]. In order to synthesize the nanocomposite microspheres, a variety of methods have been employed, such as ion exchange [26,28], photo-thermal patterning [29], block polymer micelles [30] and surface-graft processing [31]. In these methods, the synthesis of polymer and the preparation of inorganic NPs are operated individually. Thus, inorganic NPs are difficult to be well dispersed in the polymer microspheres. In addition, microemulsions [32-34] and micelles [35] are often used to prepare nanocomposite microspheres, too. Since the surfactants are difficult to be removed completely, the application of the products is limited, especially in biomedical applications [36-37]. The combination of γ-irradiation and surfactant-free emulsion polymerization can simultaneously realize the formation of microgels, reduction of inorganic ions and sterilization in the absence of surfactants, which is able to overcome the above problems and meets the request of biomedical applications. Nevertheless, because the choice of monomers is limited, the development of this method is slowly.

64
In our previous work, 4-vinylpyridine (4-VP), with the feasible coordination with metal ions [38-39], as well as its easy formation of microgel [40], has been applied to the preparation of a hybrid microgel through the simultaneous formation of Ag NPs and poly(4-vinylpyridine) (P4VP) microgel by \( \gamma \)-irradiation and surfactant-free emulsion polymerization in a single step [41-42]. It was found that the Ag NPs were well dispersed within the polymer microspheres [41-42]. However, P4VP is not biocompatible polymer. Moreover, the single commercial monomer, which can be used as the succedaneum of 4-VP, is scarce. To overcome this difficulty and obtain microgels suitable to biomedicine, we try to use several kinds of monomers to substitute 4-VP.

2.2. Research progress

In the preliminary work, poly(methacrylic acid-co-methyl methacrylate) (poly(MAA-co-MMA)) microgels was gotten via the copolymerization of methacrylic acid (MAA) and methyl methacrylate (MMA) by \( \gamma \)-irradiation and surfactant-free emulsion polymerization. Furthermore, NaOH was added into the mixed solution of MAA and MMA to neutralize part of MAA. Then, a suitable amount of AgNO\(_3\) and isopropanol were added. After being irradiated under N\(_2\) atmosphere, a brown and translucent microgel dispersion was obtained, which was stable for two months at least. In its UV-vis spectrum, there was only a single peak at \( \text{ca.} \) 410 nm, related to the surface plasma (SP) band of Ag NPs. Moreover, the symmetrical peak suggested that the circumstance around the Ag NPs was similar to great extent. The further characterization on the hybrid microgel is in process.

However, this system was not suitable to synthesize CdS-poly(MAA-co-MMA) when CdCl\(_2\) and Na\(_2\)S\(_2\)O\(_3\) were used as cadmium and sulfur sources, respectively. Because the coordination ability of S\(_2\)O\(_3^{2-}\) to Cd\(^{2+}\) is much stronger than that of methacrylate anion, there were two kinds of CdS NPs (\( \Phi: \text{ca.} 2 \) nm), \( i.e. \), one existed in poly(MAA-co-MMA) microgel (\( \Phi: 100-130 \) nm), the other distributed outside (Figure 5). Therefore, the selection of a suitable sulfur source and a monomer with a stronger coordination ability to Cd\(^{2+}\) is the most important.

\[
\text{FIG. 5. TEM image of the CdS NPs and CdS-poly(MAA-co-MMA) hybrid microgel prepared by } \gamma\text{-radiation and surfactant-free emulsion polymerization in a single step.}
\]

It was known that thiourea can coordinate with many metal ions (such as Zn\(^{2+}\), Cd\(^{2+}\)) and can generate S\(^2-\) by \( \gamma \)-radiolysis, which leads to the generation of metal sulfide NPs [43]. Therefore, we try to modify thiourea by methacryloyl group (Scheme 2). So far, the unpurified methacrylation product were obtained. After purification, the substituted thiourea will copolymerize with MAA and MMA via \( \gamma \)-irradiation and surfactant-free emulsion polymerization to synthesize CdS-poly(MAA-co-MMA) hybrid microgels.
Scheme 2

3. PUBLICATIONS

In this year, we published the following three papers, in which an acknowledgement of the contribution of the IAEA was included.


REFERENCES


[38] BRONSTEIN, L.H., SIDOROV, S.N., VALETSKY, P.M., HARTMANN, J., COLFEN, H., ANTONIETTI, M., "Induced micellization by interaction of poly(2-vinylpyridine)-block-


Adopting polyvinylpyrrolidone as template macromolecules and acrylic acid (AA) as monomers, at a concentration ranged from .05 to 1.5 %, pH sensitive nano-particle colloids were successfully prepared via template polymerization using gamma radiation in which polymerization of the monomer and self-assembly between the polymer and the template take place simultaneously. The self-assembly was driven by specific interactions between PVP and PAA produced in-situ, leading to PVP/PAAc nano-particles with insoluble inter-polymer complexes. Dynamic light scattering technique was used to indicate size shrinkage and surface charge increase of the PVP/PAAc nano-particles. Many factors affecting the PVP/PAAc nano-particle size such as irradiation dose rate, exposure dose, irradiation temperature and atmosphere, PVP MWt, and feed composition and concentration were investigated. It was found that the reactant feed composition and irradiation temperatures have a great influence on particle size of the prepared nanogel. The structure and morphology of the nano-particles were characterized by FT-IR, UV, viscometry and AFM methods. The structure stability of the nano-particles was studied at different pH solutions. The nano-particles exhibit excellent pH response. When pH changed from acid to base, the particles’ volume expanded 100 times depending on the irradiation dose at which the nanogel was prepared. The prepared nanogel was loaded with flutamide anticancer drug in the presence of ethanol-water mixture solution and the amount of loaded flutamide was determined. The prepared nano scale polyvinylpyrrolidone/polyacrylic acid bio-polymeric system loaded with flutamide drug is being investigated as anticancer target drug. Also this system will be tested for the treatment of dry-eye-syndrome.

Keywords: Nanogel, Radiation, Complex, Polyvinylpyrrolidone, pH Sensitive, Biomedical application.

1. INTRODUCTION

Recently, the interest of researchers is focused on preparation, characterization and application of nanometer-sized structural materials. There are a number of hard nano-materials, including metals [1] and ceramics [2], which have been extensively studied. Soft materials can also be engineered to form micro- and nano-structured products. Polymer gels may be categorized by their dimensions as either nano- or macroscopic gels, i.e. microgels and nanogels [3–12]. The microgel and nanogel particles are of particular interest because they exhibit their unique properties and performance which result from their small size as well as intrinsic properties of gels that combined with the properties of colloids. Each type may be selectively produced through the control of the gel-forming crosslinking reactions used to produce them. The inter-molecular crosslinking process which yields macroscopic gels has been extensively studied and is relatively well understood [13–15]. However, the radiation-induced synthesis of polymer nanogels, which is based on an intra-molecular crosslinking process, is less explored. An understanding of the mechanism of crosslink formation is very important in order to control the final, physical and chemical properties.

Recently, numerous applications of inter-polymer complexes have been described. Studies of such complexes are interesting because they are carried out in biology, medicine and biochemistry. The existence of such complexes in natural polymer plays an important function in living organisms. Nanogels based on polymers known to form inter-polymer complexes (IPC) stabilized by H-bonding can be considered as an example of one of the most promising materials. In this connection Inter-polymer complex (IPC) of polyvinyl pyrrolidone PVP proton-accepting non-ionic polymers with proton-donating polycarboxylic acids in nano-sized structure was prepared using
ionizing radiation. Comprehensive studies about formation of nano IPCs, their physico-chemical properties, and their possible biomedical applications are investigated.

2. RESULTS AND DISCUSSION

**Radiation induced formation of PVP/PAAc nano-gel.**

Trials were made to prepare PVP/AAc nanogel of different particle size using a mixture of: A- PVP / PAAc polymers, B- AAc and N-VP monomers, C- PVP polymer and AAc monomer or D- PAAc polymer / N-VP monomer. The feed solutions of same concentration and composition of reactants; PVP, PAAc AAc or N-VP were prepared using de-ionized water as a diluent and exposed to 20 kGy gamma rays of 3.3 KGy/h dose rate. The particle size of the obtained colloid solutions was determined using dynamic light scattering technique (DLS).

Poly(acrylic acid) (PAAc) is a polyelectrolyte with the proton-donating carboxyls forms inter-polymer complexes with H-accepting neutral polymers such as polyvinylpyrrolidone (PVP) as shown in the following scheme. This complex (IPC) is stabilized by hydrogen bonding.

During irradiation process, formation of inter-polymer complexes (IPC) in solutions is accompanied by irradiation simultaneous formation of many radicals on each polymer chain. These radicals undergo mainly intra-molecular recombination. In this way internally crosslinked macromolecules nanogels are formed.
FIG. 1. DLS profile of volume-weighted Gaussian size distributions of PVP/PAAc nano-particles using (A) AAc and N-VP monomers, (B) PVP and AAc, (C) PAAc and N-VP and (D) PVP and PAAc polymers. Preparation conditions: Irradiation dose of 20 kGy.
FIG. 2. DLS profile of volume-weighted Ncomp size distributions of PVP/PAAc nano-particles using (A) AAc and N-VP monomers, (B) PVP and AAc, (C) PAAc and N-VP, and (D) PVP and PAAc polymers. Preparation conditions: Irradiation dose of 20 kGy.

The particle size distributions of PVP/PAAc nano-particles synthesized by AAc and N-VP monomers system or PVP/AAc system in aqueous solution were measured by dynamic light scattering and are shown in Figs. (1, A–B and 2, A–B, respectively). The samples were added into deionized water and sonicated for 2 min. From Figs. 1 and 2 (A and B), the curve of particle size distribution of PVP/PAAc nano-particles displays a unimodal size distribution and the diameter of the particles was determined to be 85 and 250 nm for PVP/PAAc nano-particles synthesized by AAc and N-VP monomers and PVP/AAc, respectively.

For PVP/PAAc nano-particles prepared using, (PAAc / N-VP system) and (PVP/ PAAc system) (Figure 2– C and D respectively), particles showed a bimodal size distribution, a dominant peak at about 20 nm and a minor peak around 50 nm for (PAAc and N-VP system) and, a dominant peak at about 70 nm and a minor peak around 200 nm for(PVP and PAAc system). This result suggested that there is variation among particle. Thus, the system of polyvinylpyrrolidone and (acrylic acid) (PVP/AAc) was chosen. In this technique, dilute PVP solution in the presence of AAc is subjected to gamma irradiation. During the irradiation process, the first stage of the reaction is the polymerization of AAc acid into PAAc. The second stage is the formation of inter-polymer complex between PNVP and PAAc chains. During the exposure of PVP-PAAc inter-polymer complex to ionizing radiation, many tens of radicals are instantaneously formed on
every macromolecule. One of the major reaction paths of these radicals is intra-molecular recombination leading to the formation of nanogels. Radiation-induced reactions in this system shows a typical feature of intra-molecular crosslinking, i.e. a strong decrease in dimensions of a polymer coil without accompanying decrease in molecular weight. The Mechanism of PVP/PAAc nanogel formation is postulated as follows:

To confirm the formation of inter-polymer complexes between PAAc and PVP, FTIR measurements for PVP, PAAc and PVP/PAAc nanogel was performed as shown in Figure (3). It is clear that the C=O for PVP and PAAc appears at 1660 and 1710 cm⁻¹, respectively. Meanwhile, C=O for PVP/PAAc is shown at 1625 cm⁻¹ indicating the formation of inter-polymer complexes between PVP and PAAc.

To investigate the effect of various factors on the size of prepared nano-gel particles, one of the following parameters, may be changed:
1- PNVP-AAc concentration 2- PNVP/PAAc composition 3- Exposure dose 4-PVP Mwt 5- Reaction atmosphere 6- Irradiation temperature 7- Type of irradiator 8- Solvent 9- pH of the Media.

**Effect of PVP/AAc compositions**
PVP/PAAc nanogel particle size prepared at different PVP/AAc compositions and irradiation doses was determined and is shown in Figure (4). The particle size of the obtained PVP/PAAc nanogel decreases with the increasing of the PVP content in the feed solution to reach a minimum size at (30:70mol/mol) (PVP/AAc). Thereafter, any increase in the PVP content in the feed solution leads to insignificant decrease in the PVP/PAAc nanogel size.

Effect of Irradiation Atmosphere

PVP/PAAc nanogel particles were prepared at different atmosphere conditions in nitrogen, air, in the presence of H2O2. It is clear from Figure (5) that PVP/PAAc nanogel particle size prepared under nitrogen atmosphere is higher than that prepared in the presence of air or H2O2.

![Figure 4](image_url)

**Figure 4** Effect of feed composition on the particle size of the prepared nanohydrogels at different irradiation doses.

Effect of H2O2 concentration on the PVP/PAAc nanogel particle size was investigated Figure(6). It was found that by adding H2O2 (0.3 to 1.2 % v/w) to reactant feed solution, the particle size of PVP/PAAc nanogel decreases if compared with that prepared in the presence of air atmosphere feed solution. The lower the concentration of the H2O2, the lower the nanogel particle size was obtained.

The radiolysis of water under high-energy radiation is usually quite complicated and produces ionization and excitation. The molecular product hydrogen is chemically inert and readily escapes, whereas the other molecular product hydrogen peroxide H2O2, is retained in water and reacts with the reducing species (e-aq and H•) to produce OH• and corresponding species. In fact radiolysis of water in the presence of oxygen not only suppresses the reaction of recombination of the H atoms but also creates an increase in the H2O2 yield. These energy-rich species undergo dissociation, abstraction and addition reactions. As a consequence, AAc monomer undergoes chain polymerization as well as cross-linking reaction with PVP to form core-shell PVP/PAAc nano-scale particles. The termination and radical recombination reactions of polymerization and
crosslinking processes are faster in the presence of $O_2$ or $H_2O_2$ than that prepared under nitrogen atmosphere condition leading to relatively small nanogel particle size formation.

**Effect of irradiation dose**

The particle size of PVP/PAAc prepared at different irradiation dose and measured at DLS using bi-distilled water and different pH solutions was investigated and is shown in Fig. (7). There is no significant changes in the particle size of PVP/PAAc prepared at different doses and measured at low pH citrate buffer solution or bi-distilled water. However, the particle size of PVP/PAAc measured at phosphate buffer solution pH6.9 decreases with increasing irradiation dose. Thus, the prepared PVP/PAAc nano-gel is considered as pH sensitive nano-gel. At low pH the swelling of PVP/PAAc is very limited. As a consequence, the effect of irradiation dose on crosslinking content of the nano-sized PVP/PAAc is not appeared at low pH solution. At high pH the swelling behavior of nano-size prepared at different doses is very clear. As the irradiation dose increases the crosslinking density increases resulting in decreases in the swelling of the prepared PVP/PAAc nano-particles. The higher the irradiation dose, the higher the crosslinking content and the lower the swelling behavior as well as the decrease in PVP/PAAc size.

![FIG. 5. Size of PVP/PAAc nanogel particles prepared at different atmosphere conditions.](image-url)
Relative viscosity of the prepared PVP/PAAc nano-gel prepared under various irradiation doses was determined at different pHs Fig 8. The relative viscosity increases as the pH of solvated medium increases. It is also noticed that as the irradiation dose increases the relative viscosity of the prepared nanogel decreases. This means that the chains became more compact. At low pH there is strong hydrogen bonding between the polymer chains of PVP/PAAc leading to compact structure. The complexes of nanogels are characterized by lower relative viscosity than the non-complexed ones. As the pH increases, PAAc become ionized and the strong hydrogen bonding between the polymer chains of PVP/PAAc becomes weakened leading relaxed structure in the polymer chains and high relative viscosity. The more compact the structure of nanogel, the low value of relative viscosity is observed.
Effect of irradiation temperature

Figure (9) shows the effect of irradiation temperature on the mean average size of PVP/PAAc prepared at different feed compositions. The size of PVP/PAAc nanogel prepared at 15 °C is lower than that prepared at 35 °C for all feed compositions used. These results suggested that at low temperature, the concentration and recombination rate of polymer chain free radicals that responsible for the AAc polymerization and crosslinking formation of nanogel are high. As result, the termination steps for polymerization and crosslinking are faster than propagation steps, consequently, PVP/AAc nanogel of low diameter was obtained.

On the other hand, at low temperature the chain mobility of the PVP and PAAc is lower, thus the chain radicals responsible for nano-particle formation close each other and the probability of the radicals to combine and to form covalent bonds between the nano-particle chains is higher than that at high temperature. As a result, nano-particle of relative small size is formed if compared with that formed at high temperature.
Effect of PVP MWt

Effect of Different MWt of PVP (8000, 40000 and 130000) on particle size of PVP/PAAc prepared by ionizing radiation at different feed solution compositions was investigated Figure (10). Irradiation feed solution of PVP/AAc containing 8000 Mwt PVP, resulted in PVP/PAAc aggregated copolymer. However, the nano-sized PVP/PAAc gel was obtained when 40000 and 130000 MWt PVP is used. Meanwhile, the mean size of PVP/PAAc hydrogel obtained from 130000 Mwt PVP is lower than that obtained from 40000 MWt PVP. Insignificant changes in mean diameter of PVP/AAc nano-size resulted from irradiated feed solution containing (1300000) MWt PVP with different doses was observed. However the mean diameter of PVP/AAc nano-size resulted from irradiated feed solution containing (40000) MWt PVP increases with increase irradiation dose (the data is not shown).
Effect of irradiator type and Dose rate on the PVP/PAAc particle Size

PVP/PAAc colloidal particles were prepared using electron beam irradiator and gamma ray irradiator. It was found that there is no great difference in PVP/PAAc nano-particle size prepared by 20 kGy gamma rays of 3.85 K Gy h−1 dose rate and that prepared at the same exposure dose using electron beam of speed, 3.6 m min−1. Meanwhile, for the gamma irradiator, as the irradiation dose rate increases the particle size decreases. This can be attributed to that at high dose rate the radical formed is higher and probability of these radicals to recombine and terminate is high. As a result, short polymer chains and small PVP/PAAc particle size is formed (Figure 11).

Transmittance of PVP/PAAc nano-gels

Changes in the transmittance of PVP/PAAc nano-gels prepared at different PVP/AAc compositions recorded at 500 nm are illustrated as a function of pH of PVP/PAAc nanogel solutions, Figure (12). Value of critical pH is calculated as a crossing point of lines that come through experimental points before and after significant change of curves.
Critical pH values for PVP/PAAc nanogel prepared from feed solution of composition (0.7%:0.8%) and (0.3%: 1.2%) (PVP: AAc) are 5.6 and 4.7 respectively. One of the reasons for a difference in pH-crit. may be due to the average pKa. A stronger complex was formed at stoichiometry that deviates from equimolar and as results of complex formation, the pHcrit increases. Thus, the complex formation between PVP and PAAc from feed solution of composition (0.7%:0.8%) (PVP: AAc) is stronger than that obtained from (0.3%: 1.2%) (PVP: AAc) feed composition.

FIG. 11. Effect of irradiation dose rate on the PVP/PAAc particle Size.

FIG. 12. Changes in the transmittance of PVP/AAc nanogels prepared at different PVP /AAc compositions recorded at 500 nm.
**pH-responsive behavior of PVP / PAAc nanoparticles**

The obtained PVP / PAAc colloid nano-particles are highly stable. There was almost no change in the size or precipitation of the prepared colloid nano-size particle after storing for 60 days. However, the colloid nano-particles showed un-stability and aggregation when the pH of the nanogel particle solution is less than 3.5.

**FIG. 13. pH-Responsive behavior of the PVP-PAAc nanogel particles prepared at different irradiation doses.**
The PVP / PAAc nano-particles are co-stabilized by the electrostatic and hydrogen bonding interactions, which closely depend on pH of the medium. When pH value decreased above 3.5, the surface charge of the particles decreased to be too low to stabilize the particles, and obvious aggregations between the particles began to appear. Therefore, it is interesting to study the pH-responsive behavior of the nano-particles, as shown in Figures (13-14). To investigate the effect of medium pH on the PVP-PAAc nanogel particles, the particles prepared at different irradiation dose were incubated in a series of buffer solutions with different pH values (pH 3.5, to 7.8). The particles’ size increased rapidly with increasing pH value from 4.5 to 5.5 until reaching a maximum at pH about 6, and then they increased slightly. At a constant pH, as the irradiation dose highly increases, the swelling of the nanogels becomes limited and their size is diminished. At different pH values, the increase in gel particle size that prepared at 40 kGy ranged from 100 to 140nm (nano-gel size expands by 40%). Meanwhile, the particle size of the gel irradiated at 10 kGy increases from 130 to 260nm (nano-gel size expands by about 100%) Figure 13.

At low pH, the contraction in the particles is due to the hydrogen bonding formation between COOH groups. As pH increased, the size of particles increased due to the repulsion between the COONa groups of the particles, causing particle size stability. However, at one particle the repulsion between the COONa groups increases resulted in increase in water penetration inside the particle leading to the initial size increase. This monotonic expansion in the size may be caused by the disassociation of inter-polymer hydrogen bonding and stronger electrostatic repulsion generated by the ionization of the PAAc chains in the particles due to the increase of pH. On the other hand, in the region of pH 5.8, little change in the size of the particles has been found with the increase of the pH value. This is due to that PAAc molecules have been totally ionized at pH 5.8.
The resulting zeta potential of the particles at different pH values is shown in Figure 14. The zeta-potentials of the PVP-PAA nanogel particles also reveal the same pH size change trend. It can be seen that at pH 4, the zeta-potential is -27mV. However, at pH 4.7, the hydrogen bonding between the PVP and PAA begins to be weakened because about 50% of carboxyl groups in PAAc chains are deprotonated, resulting in a negative-potential 35 mV appears. When pH is larger than 5.8, the potential reaches -50 mV. These results are well correlated with the changes in particle size, suggesting that the gel particles are pH-sensitive.

**Morphology of PAA/PVP nano-particles**

AFM is a powerful tool for observing morphologies of PAA/PVP nano-particles. As shown in Figure 15, it could be barely seen, without staining, slightly deformed spherical particles with size about 140-180 nm.

**Drug loading**

PVP/PAAc nano-gel prepared at 20kGy was loaded with flutamide in the presence of ethanol-water solution. The ethanol was evaporated and the unloaded flutamide was precipitated. The amount of loaded flutamide can be determined. AFM was used to observe morphologies of nano-gel loaded with flutamide Figure 16. Spherical particles of PAA/PVP nano-particles loaded with flutamide was noticed (200-300 nm). Also, it appears that the average diameter of drug-loaded nanogel is larger than that of blank one Figure 15. This is because flutamide was entrapped into the nano-particle by electrostatic and hydrogen bonding interactions, which will result in the little increase of nanogel size. The nano polymer loaded with drug is under investigating for using as anticancer target drug. This nano-scale PVP/PAAc bipolymeric system will be tested for the treatment of dry-eye syndrome.

![FIG. 15. AFM OF PVP/Ac nanogel prepared at 0.3% PVP – 1% AAc Dose: 20 kGy, pH 5.](image)
3. CONCLUSION

Nanogel particles can be easily prepared using PVP-AAc system. The particle size of the prepared PVP-PAAc nanogel can be controlled through: concentration and composition of feed solution, irradiation dose, irradiation temperature, irradiation dose rate, PVP Mwt, and oxygen atmosphere. The size of the prepared PVP-PAAc nano-particles can be determined using different techniques AFM, DLS, TEM and q nano apparatus. The size of the prepared nano-particles is highly affected by the medium pH. The prepared PVP-PAAc Nano particles can be used in biomedical applications.

REFERENCES


SYNTHESIS OF SPECIFIC NANOPARTICLES FOR TARGETING AND IMAGING TUMOR ANGIOGENESIS USING ELECTRON-BEAM IRRADIATION

G. Rizza, S. Deshayes, V. Maurizot, M.-C. Clochard, T. Berthelot, C. Baudin, G. Déléris; FRANCE

1. INTRODUCTION

Angiogenesis, the formation of new capillary blood vessels from pre-existing vasculature, plays an essential role in normal processes, such as embryogenesis, wound healing and in pathological processes like tumor growth (Heljasvaara et al, 2005). Inhibition of angiogenesis represents then a promising strategy to block tumor growth and invasion. A number of endogenous angiogenic regulators such as VEGF, fibroblast growth factor (FGF) and angiopoietins have been identified (Zilberberg et al, 2003). VEGF and its receptors (VEGFR-1 and VEGFR-2 which are tyrosine kinase activity) are frequently up-regulated in a number of clinically important human diseases, including cancer, making them an attractive target for therapies (Miao et al, 2006). Different strategies have been designed to inhibit VEGF function by blocking its interaction with its receptor (Keedy et al, 2007; Zilberberg et al, 2003). A 17-amino acid cyclo-peptide was previously described as a vascular growth inhibitor (CBO-P11) (Zilberberg et al, 2003). This molecule encompasses residues 79-93 of VEGF which are involved in the interaction with its receptor and shows a micromolar affinity for VEGFR-2.

In order to improve circulation time of the peptide, nanoparticles may be used for the transportation of the drug to the target tissue. Nanoparticles most commonly refer to solid colloidal particles made of macromolecular material ranging from 1 to 1000 nm. They can be used as drug carriers, either by dissolving, entrapping, encapsulating or attaching the active substance. Various types of carriers have been developed such as polymeric micelles, polymer-based nanoparticles and liposomes (Van Butsele et al, 2007). Therapeutically used polymeric nanoparticles are composed of biodegradable hydrophobic polymers protected by an amphiphilic block copolymer that stabilizes their dispersion in aqueous media (Sung et al, 2007; Van Butsele et al, 2007). With regard to the hydrophobic core, we are interested in a fluorinated polymer, poly(vinylidene fluoride) (PVDF). This latter is a semicrystalline thermoplastic, biocompatible polymer, remarkable for its physical and chemical resistance. In addition to numerous industrial applications, PVDF shows new interests in biotechnology (microporous and ultrafiltration membranes) and in biomedical activity (vascular sutures, regenerated templates) (Braga et al, 2007; Chen et al, 2006a; Chen et al, 2006b; Marchand-Brynaert et al, 1997). However, no literature to our knowledge reports on the use of PVDF nanoparticles as a carrier for drug delivery.

The aim of the present paper is to immobilize of a bioactive peptide such CBO-P11 onto PVDF nanoparticles. PVDF presents a high hydrophobicity. Therefore, to improve its hydrophilicity, the nanoparticles were coated with poly(acrylic acid) (PAA) using electron-beam irradiation to obtain a grafted copolymer, PVDF-g-PAA (Betz et al, 2003; Clochard et al, 2004). PAA carboxylic acid functions allow the coupling of a spacer arm to occur on nanoparticles and CBO-P11 was covalently linked to the spacer arm by click chemistry reaction (Scheme 1). Every step has been characterized by HRMAS NMR or MALDI mass spectrometry.
2. EXPERIMENTAL PART

Synthesis of PVDF nanoparticles: The synthesis has taken place at PiezoTech SA. Poly(vinylidene fluoride) nanoparticles were prepared in a reactor under pressure by nanoemulsion polymerization of corresponding monomer (VF₂) as described in a previous paper (Kappler et al, 2004). The monomer is emulsified in a continuous phase of water using potassium persulfate as initiator and perfluorooctanoic acid as ionic surfactant. These perfluorinated surfactants promote micellization at low concentration (Moody et al, 2000). To stabilize the emulsion, paraffin wax was used as dispersing agent.

Irradiation: Samples of lyophilized PVDF nanoparticles were put inside sealed glass tubes under vacuum. Irradiations were performed at room temperature using a 10 MeV Pulsed Electron Beam industrial accelerator at Ionisos (Chaumesnil, France). Irradiation doses lies in range of 25 to 200 kGy.

Grafting: The irradiated powder of lyophilized PVDF nanoparticles was dispersed into a grafting aqueous solution of AA. Grafting experiments were performed at 60°C for 1 h. At this temperature, two chemical reactions occur: i) thermal homopolymerization of AA, and ii) grafting reaction on the nanoparticles. Therefore, to avoid homopolymerization, we have added 0.25 wt% of Mohr’s salt (Scheme 1). The nanoparticles were purified by centrifugation and dialysis. Finally, they were freeze-dried to obtain a white powder of copolymer (PVDF-g-PAA [1]).

PVDF-g-PAA nanoparticles decoration: mTEG was coupled to [1] via an amide bond to the carboxylic acid function of PAA using ethyl-3(3dimethylaminopropyl)carbodiimide (EDC) in an aqueous solution at room temperature for 24 h (Scheme 1). The nanoparticles were purified by centrifugation and dialysis. Finally, they were freeze-dried to obtain a brown powder (PVDF-g-PAA-mTEG [2]). CBO-P11 or cyclo-VEGI (D-FPQIMRIKPHQGHIGE) was synthesized by Fmoc/t-Bu batch solid-phase synthesis (Goncalves et al, 2005). After cleavage from the resin and before deprotection of the peptide, propargylamine was coupled to the glutamic acid unit. Finally, the peptide was deprotected and purified by reversed-phase HPLC. Then, the purified peptide [3] was coupled to [2] by click chemistry using copper sulfate and sodium ascorbate at 40°C for 3 days. The nanoparticles were purified by centrifugation with an EDTA solution in order to remove copper. The supernatant was collected, purified by reverse-phase HPLC in order to quantify unreacted peptide and to determine indirectly the grafting yield. Then, the nanoparticles were dialysed and freeze-dried to obtain PVDF-g-PAA-mTEG-P11 [4] as a white powder.

Field Emission Scanning Electron Microscope (FESEM): A Hitachi S-4800 field emission scanning electron microscope equipped with a tip made of Zr monocrystal allowed us to take pictures of the fragile PVDF nanoparticles without metallization. Accelerating voltage: 1kV. Tip current : 10 µA; Probe current: Normal; UltraHigh Resolution Mode; Condenser lens : 5; Focus depth : 1; Objective lens diaphragm : 2 ; Working distance : 2 mm.

Dynamic light scattering (DLS): A Zetasizer Nano-ZS dynamic light scattering (Malvern instrument 3000HSA) was used for nanoparticles characterization.

Small Angle Neutron Scattering (SANS): Measurements were performed on PACE spectrometer at LLB (CEA-Saclay). Nanoparticle latexes were diluted 10 times in D₂O. For each measurements, neutron scattered intensity was recorded as a function of scattering vector. Data treatment was performed following a previous paper (Brulet et al, 2007). Scattered intensity profile is accounted for using the form factor of a sphere.

Electron Paramagnetic Resonance (EPR): EPR spectra were recorded at the X band (9.4GHz) on a Bruker ER-200D EPR spectrometer.
3. RESULTS AND DISCUSSION

Characterization of PVDF nanoparticles
The particle size was determined by FESEM, by Zetasizer Nano-ZS dynamic light scattering (DLS) (Malvern instrument 3000HSA) and by small angle neutron scattering (SANS). Results obtained by these different characterization methods are similar. The radius of nanoparticles is around 60 nm with a polydispersity index of 0.002 determined by DLS, indicating a monodisperse latex. A typical FESEM image is shown in Fig. 1. PVDF nanoparticles appear spherical and monodisperse.

Radical stability
Under vacuum, electron beam irradiation generates mainly alkyl radicals inside the PVDF polymer bulk. When opening the irradiation tube, the PVDF nanoparticles come in contact with air. Alkyl radicals combine immediately with oxygen to form peroxide radicals. The radical amount is proportional to the absorbed dose. If the irradiation dose is important, a yellowish colour appears corresponding to unsaturated bound creation. Figure 2 shows EPR spectra at different doses. The hyperfine band at 3520 Gauss observed at 200 kGy is due to polyenyl formation. The EPR spectrum corresponding to irradiation dose of 50 kGy of a sample opened for 3 months is similar to the one corresponding to irradiation dose of 25 kGy. Nanoparticles radical content is consequently far to be stable with time. This behaviour is different from what it has been already observed for PVDF film (Clochard et al, 2004). The high stability in films is assumed coming from radical trapping inside the polycrystalline part of the PVDF (Aymes-Chodur et al, 1999; Aymes-Chodur et al, 2001; Clochard et al, 2004). A DSC study (Fig. 3) allows us to determine the crystallinity rate of the nanoparticles at various doses. For a 100% crystalline PVDF, \( \Delta H_{\text{PVDF 100\% crystalline}} \) is equal to -25 cal/g or -104.5 J/g. Nanoparticles enthalpies were found equal averagely to \( \Delta H_{\text{PVDF nanoparticle}} = -29.5 \) J/g whatever the irradiation dose and \( \Delta H_{\text{PVDF film}} = -38 \) J/g corresponding to 28 ± 2 % et 36 ± 2% crystallinity rates respectively. Results show a diminution of crystallite content for nanoparticles in comparison with films. Moreover, the major variation observed in DSC curves (Fig.3) is the shift in melting peak to lower temperature. Indeed, the melting temperature (Tm) is at 160°C for the nanoparticles and at 167°C for the films. It means that the crystallite size is much smaller in nanoparticles. Electron beam irradiation seems to not affect the crystallinity rate of nanoparticles. Consequently, the radical decay with time in nanoparticles may be due to small crystallite content. The high specific area of nanoparticles may also participate to this radical consumption. Indeed, the available area for oxygen to combine with alkyl radicals is huge compared to a flat film and peroxide radicals are well-known to be less stable. Consequently, it is of great importance to radiograft quickly after irradiation.

Radiation Grafting of PVDF with acrylic acid.
Fig.4 shows proton NMR spectra of PVDF and PVDF-g-PAA [1] recorded in DMF-\( d_7 \). In PVDF spectrum, the large triplet at 3.03 ppm corresponds to the signal of CH\(_2\) repeat unit and the triplet at 2.5 corresponds to CH\(_3\) signal of PVDF chain ends. The PVDF-g-PAA spectrum displays large peaks at 1.98 and 1.73 ppm corresponding to the CH\(_2\) signal of PAA. CH of PAA gives rise to a signal at 2.5 ppm. From integrated signals, a quantitative approach allows us to evaluate the PAA/PVDF ratio. The resulting yield for PVDF-g-PAA is found equal to 56 mol%.
Covalent coupling of spacer arm.

A spacer arm onto nanoparticles brings mobility to the future immobilized peptide. We synthesized a modified tetraethylene glycol (noted mTEG) with an amine function at one end and an azide function at the other end. This linker was chosen because of its solubility and a previous study shows that this spacer arm did not affect the activity of CBO-P11 (Goncalves et al, 2005). The HRMAS NMR spectrum of [2] is shown in Fig.4 and displays two peaks for CH$_2$ of the spacer arm (CH$_3$CH$_2$O and CH$_2$NH) at 3.63 and 3.67 ppm. On the other hand, the signal corresponding to CH$_2$N$_3$ is masked by the water peak at 3.46 ppm. Integration of CH$_2$ (mTEG) signal allows us to evaluate the mTEG/PVDF ratio and to determine a 31 mol% yield for PVDF-g-PAA-mTEG. It means that only 50% of the available carboxylic acids were covalently bound to spacer arm.

Peptide synthesis and coupling to nanoparticles by click chemistry

In order to attach the peptide CBO-P11 onto nanoparticles, a functionalization of the original peptide is needed. Propargylamine was coupled to the glutamic acid unit in order to have an alkyne function (Scheme 1). This later allows further anchoring via click chemistry reaction to the spacer arm without affecting other side chains of the peptide which are essential for the recognition with VEGFR-2 receptors. The peptide coupling was proved by MALDI mass spectrometry. The analysis of nanoparticles was performed in positive mode with Voyager STR mass spectrometer (Applied Biosystems). The signal at $m/z$ 1998 Da corresponds to the CBO-P11 mass. It indicates the peptide grafting. From the supernatant-removed peptide, the grafting yield for PVDF-g-PAA-mTEG-P11 [4] was found equal to 5.5 mol%.

4. CONCLUSION

We have succeeded to synthesize PVDF nanoparticles by nanoemulsion polymerization and their functionalization with a peptide that presents an anti-angiogenic activity. Resulted nanoparticles present a radius of 60 nm. From FESEM images and light scattering measurements, we deduced that they were spherical and monodisperse. The alkyl radicals induced from electron beam irradiation combine immediately with the oxygen to form peroxide radicals. Because of a high specific area and small crystallite size, the radical decay with time is evidenced from EPR measurements. Despite this radical decay, electron beam irradiation allows us to graft PAA by radical polymerization onto freshly irradiated PVDF nanoparticles and then to immobilize CBO-P11 by click chemistry via a spacer arm. Evidences of grafting were shown using HRMAS NMR and MALDI-TOF mass spectrometry. Nanoparticles functionalized with an angiogenesis-targeting agent are an attractive option for anti-tumor therapy.

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REFERENCES


Scheme 1. Reaction scheme of PVDF-g-PAA-mTEG-P11 synthesis.
FIG. 1. FESEM image of PVDF nanoparticles recorded without metallization at 1000V.
FIG. 2. EPR spectra of PVDF nanoparticles irradiated under vaccum and put in contact with air for 5 minutes (25 kGy and virgin200 kGy) and for 3 months (50 kGy).
FIG. 3. DSC curves of PVDF virgin films and irradiated nanoparticles.
FIG. 4. HRMAS 500 MHz NMR proton spectra of PVDF (---), PVDF-g-PAA (---) and PVDF-g-PAA-mTEG (—) in DMF-d$_7$. ● and ○ correspond to DMF and water residual solvents respectively. HRMAS NMR experiments were carried out on a Bruker Avance 500 MHz equipped with an HRMAS dual probe ($_1^1$H/$^{13}$C) and 4 mm HRMAS rotor (ZrO$_2$).
POROUS POLYMER DRUG-ELUTING COATING PREPARED BY RADIATION INDUCED POLYMERIZATION

M. Veres, B. Beiler, L. Himics, S. Tóth, M. Koós; HUNGARY

1. INTRODUCTION

Many areas of modern medicine are almost unimaginable without the use of different kinds of implants. They used as replacements, supports, auxiliary devices etc. for various parts or functions of the body. Their use has many advantages, however there could be some drawbacks too, like the possibility of rejection, inflammation and other side-effects. Many of these drawbacks are directly related to the materials used for the implant fabrication. Coatings are widely used to eliminate the unwanted effects appearing after the implantation. In addition to the protection and separation of tissues from the implant material they could also enhance the functionality and the acceptance of the artificial device and also promote the regeneration of the tissues after the intervention. Drug-eluting coatings are a good example for the latter. By delivery and controlled elution of drugs they could actively suppress inflammatory reactions, allergy and rejection of the implant, and their activity is localized to the place where these effects could mainly occur – to the region of the implant. This project is aimed to develop a drug-eluting porous polymer coating by radiation induced polymerization that can be used in different medical implants. The primary objects for this research are coronary stents however these porous layers could have perspective in other types of medical devices too. The main objectives are to develop a method for coating the surface of medical grade metallic alloy wires, plates and tubes with a porous polymer nanocomposite layer prepared by radiation induced polymerization and to characterize the obtained coatings.

2. MATERIALS AND METHODS

2.1. Materials

Diethyleneglycol dimethacrylate (DEGDMA, Aldrich) and 2-hydroxyethyl-methacrylate (HEMA, Aldrich) monomers were used for the preparation of the coatings. Ethanol, methanol, propanol, glycerol, acetone and ethyl acetate (all were purchased from Aldrich) were used as solvent materials. Amorphous carbon films were prepared by using 5.0 methane and argon gases (Linde).

The stainless steel (SS) wire, plate and tube supports were made of medical grade 316LVM or Inconel alloys widely used in medicine for the preparation of different implants, including stents.

2.2. Methods

The polymers were prepared by $\gamma$-radiation induced polymerization method. In order to avoid the contamination from the atmosphere, the whole sample preparation procedure was carried out under Ar atmosphere in a MBraun Unilab glovebox workstation. Alloy substrates were used in form of 1-2 cm long wires of 130 micron thickness, 500 micron thick plates having size of 1 cm x 2 cm and tubes with 1.25 mm o.d. and 300 micron wall thickness. These were either mounted into the rubber sealing of the vial caps (wires) or simply put into the vials (plates and tubes) containing the
monomer mixture. In the case of multi-layer systems the samples did not contact with air during the transfer to the vial with the second monomer mixture. The irradiation was carried out at room temperature, the absorbed dose was 1-20 kGy, and the dose rate was 7 kGy/h.

The amorphous carbon (a-C:H) film deposited on some of the alloy substrates was developed by us earlier as a protective layer for stents [1-3]. These a-C:H layers were deposited using radio frequency chemical vapor deposition from methane precursor gas and have thickness of a few hundred nanometers.

The morphology of the obtained coatings was investigated using a Leica DM/LM optical microscope, and JEOL JSM-740 and Hitachi S4700 scanning electron microscopes (SEM). The bonding configuration of the samples was characterized by Raman spectroscopy on a Renishaw 1000 micro-Raman spectrometer. A 27 mW diode laser operating at 785 nm served as excitation source. The excitation beam was focused into a spot having diameter of 1 micron. Conversion of the polymers was determined from the mass difference of the monomer and the obtained polymer.

3. RESULTS

Development of porous DEGMA coating

The prerequisite of the formation of a uniform porous polymer layer on the stainless steel wire is the coating of its surface with a uniform film of the polymerization mixture that remains there during the whole polymerization process. In this aspect the surface properties of the substrate, wetting characteristics and viscosity of the monomer mixture are the key parameters that will determine the formation of the polymer coating on the implant surface. Several different approaches were tried to influence the coverage of the stainless steel surface by the monomer mixture, including the use of different solvents and solvent mixtures suitable also to form porous polymers [4,5] in order to improve the wetting, additives to increase the viscosity of the mixture, spin coating and dip-coating techniques.

Pre-polymerization was also used with the aim to increase the density of the mixture before applying it to the substrate. In this case the mixture was irradiated with small doses (1-2 kGy) in order to form small polymerized fragments in the monomer mixture which would thicken it. The obtained mixture had higher viscosity, but because of the specific features of the polymerization, this did not meant a better coating of the substrate surface. Formation of porous DEGDMA polymers (monoliths) is based on the different solubility of the monomer and the formed polymer in the solvent [4]. Porous structure will form if the solvent is a good solvent for the monomer and bad solvent for the polymer. In this case the formed polymer nuclei will precipitate soon after the polymerization starts. Further polymerization will take place mainly in the surrounding of these precipitated nuclei (at the interfaces) and the forming polymer chains will attach to these nuclei creating globules that will grow and coalesce with other globules with time. These interconnected units constitute the frame of the porous polymer. The observed thickening of the mixture during pre-polymerization was due to the precipitation of the formed globules. However they still were surrounded by the (bad) solvent that prohibited their attachment to the alloy surface.
In order to increase the viscosity of the initial mixture, glycerol was added to it. Preliminary experiments were carried out to prepare the porous polymer in bulk by irradiating 40 vol.% DEGDMA dissolved in pure glycerol. However this resulted in formation of a transparent material in the top part of the mixture, indicating that either the DEGDMA content was too high or the glycerol as solvent is not suitable for the preparation of the porous polymer. The dense liquid found in the bottom of the vial pointed on that in spite of the stirring even right before the irradiation the monomer and the solvent separate relatively quickly and a DEGDMA-rich solution formed (and has been irradiated) in the upper part of the container, while almost pure glycerol remained in the bottom. To decrease the viscosity and to obtain better homogeneity of the solution, ethanol was added to the polymerization mixture in different concentrations. Trialing several different ethanol/glycerol ratios it was found that the polymerization mixture containing 60 vol.% solvent (with ethanol/glycerol ratio of 1:1) and 40 vol.% of DEGDMA has the required characteristics and well-suited for coating purposes.

Fig. 1a shows the optical microscopic image of a stainless steel wire coated with the porous polymer formed by irradiation of the mixture of 30 vol.% ethanol, 30 vol.% glycerol and 40 vol.% DEGDMA. It can be seen that a continuous opaque layer formed on the surface having thickness of a few microns. SEM analysis (Fig. 1b) showed that the layer has a fractal structure and is composed of coalescing sub-micron sized polymer globules.

![Figure 1a](image1.jpg) ![Figure 1b](image2.jpg)

**FIG. 1.** (a) Optical microscopic image of stainless steel wire coated with the porous polymer. (b) Morphology of the porous polymer layer.
Figure 2 shows SEM images of the formed polymer nuclei attached to the wire surface. It can be seen that aggregated polymer globules are distributed non-uniformly on the surface, implying that the presence of active sites is required on the metal surface, that serve could as precipitation centers where the polymer globules could attach. The aggregation of the particles into clusters on the surface is characteristic for fractal structures. These results indicate that the surface quality of the support is critical for obtaining uniform layer and good adhesion.

In parallel to the bare metal surfaces steel wires with amorphous carbon coatings were also used as substrates (Fig. 3). However formation of continuous layer was not observed on these samples. As SEM analysis showed, polymer globules were attached only in some places to the amorphous carbon surface, and the coverage was much less than for bare metal substrates. However in those places a large number of particles coalesce forming relatively large clusters of globules on the carbon surface. It seems that the properties of the hydrophobic amorphous carbon surface does not promote the formation of poly-DEGDMA layer on top of it.

The above results show that it is possible to prepare a porous polymer coating on stainless steel surface that can be utilized as drug container, but it will lack of uniformity and full coverage of the implant surface. Because of the porous character of the coating for use in implants an additional protective bottom layer is required. But the DEGDMA layer on systems with a-C:H film had worse properties than on bare metal surfaces. Therefore we decided to modify the design of the coating to
a composite layer, in which the porous polymer “drug containers” are embedded into a host matrix made.

*FIG. 4. SEM micrographs of (a) bare Inconel surface, (d) a-C:H coated Inconel surface; HEMA hydrogel on (b, c) metal and (e, f) amorphous carbon.*

**HEMA/DEGMA composite coatings**

In addition to the uniformity and coverage it could provide, the host matrix would have another remarkable advantage – by tuning the porous properties it could be used for the control of the drug elution characteristics of the coating [6]. Hydrogels are ideal materials for such purposes. Therefore the host matrix was prepared from 2-hydroxyethyl methacrylate, a biocompatible material used in medical devices, that can easily be prepared by radiation induced polymerization and has the
required mechanical properties too. The monomer mixture composed of 60 vol.% water, 35 vol.% HEMA and 5 vol.% DEGMA cross-linking agent [7].

![Image](image1.png)

**FIG. 5.** Wetting of the (a) bare Inconel alloy surface, (b) bare metal surface after Ar plasma treatment and (c) stainless steel with a-C:H coating by the monomer mixture.

Both bare alloy plates and coated with amorphous carbon metal surfaces were tried as substrates for the hydrogel coating. Formation of the polymer was observed on both substrates (Fig. 4). But the detailed analysis showed that it is more uniform and continuous on a-C:H (Fig. 4, e and f) than on the bare metal surface. Spherical microglobules of hydrogel can be seen on the latter (Fig. 4 b and c) indicating the clotting of the material. This is caused by the difference in wetting properties of the two surfaces by the monomer mixture (Fig. 5), which, in case of metal, could be improved by Ar plasma treatment, but it is still far from the almost perfect wetting found for a-C:H.

The presence of the hydrogel on a-C:H surface was proved also by Raman spectroscopy (Fig. 6). Characteristic peaks of HEMA hydrogel can clearly be seen on top of broad a-C:H bands. Comparison of the spectra of bulk and deposited on a-C:H surface hydrogel shows that the amorphous carbon has no effect on the structure of the hydrogel.

![Image](image2.png)

**FIG. 6.** 785 nm excited Raman spectra of (a) bare metal substrate, (b) the hydrogel, (c) a-C:H layer on metal substrate and (d) HEMA hydrogel on top of a-C:H. The relatively low intensity of the polymer peaks compare to those of a-C:H is due to the significantly lower scattering cross-section of the former.
FIG. 6. SEM images of (a and b) aggregated DEGDMA microglobules on the HEMA hydrogel surface and (c and d) DEGDMA nanoparticles embedded into HEMA hydrogel.

HEMA/DEGDMA composites were also fabricated by subsequent coating and irradiation of the already formed HEMA layer with DEGMA monomer mixture and also by a one-step process using a monomer mixture with excess DEGDMA content. In the first case aggregated DEGMA microglobules were formed on the hydrogel surface (Fig. 6 a and b), that were similar to those observed on a-C:H coating (Fig. 3). Embedded DEGDMA nanoparticles (with average size below 100 nm) were observed as a result of the one-step process (Fig. 6 c and d).

**Polymerization of DEGDMA**

The pre-polymerization experiments with different doses and the trials with different solvents allowed to investigate the effect of the dose and solvents on the formation and bonding configuration of the porous polymers. Fig. 7 shows a typical evolution of the Raman spectra of DEGDMA polymers with dose. The spectra look very similar, but there are some features changing in good correlation with the applied dose. The intensity of the peaks at 1405, 1640 and 1723 cm⁻¹ decrease with the increasing dose.
Raman spectroscopy can be used for the characterization of the conversion of polymers [8,9]. If the polymerization takes place by braking of C=C bonds followed by interconnection of the monomers, the intensity of C=C peaks of the monomer would be a good indicator of the conversion, being inversely proportional to that. In case of DEGDMA the C=C peaks can be observed at 1405 and 1640 cm\(^{-1}\), and these peaks really decrease with the dose (Fig. 7). Fig. 8 compares the conversion of DEGDMA monomers in different solvents obtained by mass difference measurements with the evolution of the intensity of C=C Raman band at 1640 cm\(^{-1}\). Left panel shows that the fastest conversion was obtained in alcohols, while the use of ethyl acetate and acetone results in slower polymerization. With alcohols the conversion is already almost 100% at 8 kGy dose. The intensity of the Raman bands follows well the
FIG. 8. Conversion of DEGDMA monomer in different solvents. Left panel: conversion data obtained from mass difference measurements; right panel: dependence of the intensity of the C=C peak at 1640 cm⁻¹ with dose.

FIG. 9. Effect of DEGMA concentration on the intensity of C=C Raman peak and stiffness of the resulting polymer.
conversion curves (right panel), except that it doesn't equal to zero even at 15 kGy dose, where the conversion was found to be 100%. The presence of the peak in the spectrum of these samples indicates intact C=C bonds, which can be due to monomer molecules trapped inside the matrix or “dangling” monomer molecules that bond to the matrix only through one bond. Increase of the DEGDMA content of the monomer mixture results in lowering of the C=C peak intensity in the spectra (Fig. 9), indicating that these structures contain less amounts of the above species. The higher monomer content has also a significant effect on the mechanical properties of the polymer (Fig. 9).

4. SUMMARY

Porous polymer DEGDMA drug-eluting coatings have been prepared on medical grade metallic alloys by radiation induced polymerization. The required coverage of the metal surface by the monomer mixture was achieved by using ethanol/glycerol solvent mixture.

New HEMA/DEGDMA composite polymer layers have also been developed, that can be used as drug delivery coatings on medical implants. They can be prepared either in form of multi-layer HEMA-DEGMA structures by a multi-step irradiation polymerization process or in a single step by using HEMA/DEGDMA monomer mixture with increased DEGDMA content. In the latter case the resulted material contained porous DEGDMA nanoparticles embedded into the HEMA hydrogel matrix.

Raman spectroscopic analysis of the DEGDMA polymerization with irradiation dose showed that intact C=C bonds remain in the polymer even after 100% conversion, indicating the presence of trapped or partially polymerized monomers in the matrix. Their amount decreases with the increase of the DEGDMA concentration in the monomer mixture, which also influences the mechanical properties of the formed polymer.

Based on the CRP we started a collaboration with the group of Prof. Kwang-Pill Lee (South Korea) and in 2009 we got a 2-year support from the Hungarian-South Korean Intergovermental S&T Programme. The aim of our common work is to prepare and characterize functionalized nanocrystalline diamonds by radiation methods.

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REFERENCES


POLY (LACTIC ACID)/LAYERED SILICATE NANOCOMPOSITE FILMS: EFFECT OF IRRADIATION

S. Dadbin, F. Naimian, A. Akhavan, S. Hasanpoor; IRAN

Summary
Poly (Lactic acid) –layered silicate nanocomposite films were prepared by solution casting method. The films were irradiated with Co \(^{60}\) radiation facility at dose of 30 kGy. The effect of gamma irradiation on mechanical properties of the neat PLA and nanocomposites was evaluated by data obtained from tensile testing measurements. The tensile strength of the irradiated PLA films increased with addition of 1 wt% Triallyl Cyanurate (TAC) indicating crosslink formation. Significant ductile behavior was observed in the PLA nanocomposites containing 4 pph of nanoclay. Incorporation of nanoclay particles in the PLA matrix stimulated crystal growth as it was studied by differential scanning calorimetry (DSC). The morphology of the nanocomposites characterized by transmission electron microscopy (TEM) and X-ray diffraction (XRD) revealed an exfoliated morphology in the PLA nanocomposite films containing 4 pph of nanoclay. Only very small changes were observed in the chemical structure of the irradiated samples as it was investigated by Fourier transform infrared (FTIR) spectroscopy. Enzymatic degradation rate of PLA and its nanocomposite decreased with increasing crystallinity of the samples. The rate of weight loss was also affected by the morphology of the nanocomposites

Key words: biodegradable, biomaterial, poly (lactic acid), nanocomposite, \(\gamma\)-irradiation

1. INTRODUCTION
Poly (lactic acid) (PLA) is a linear aliphatic thermoplastic polyester. Good mechanical properties along with its biocompatibility make it suitable for biomedical applications [1]: such as prosthetic implants [2], three dimensional scaffolds [3], controlled-release drugs [4] and resorbable sutures. There is also a growing interest in utilizing PLA for packaging and disposable plastic articles since production cost has been lowered by new technologies and large scale production [5-6]. In spite of many good properties of PLA which makes it a preferred choice for substitution with petroleum-based polymers, existence of a few weaknesses in some aspects such as thermal stability, barrier properties and toughness seeks a requirement for its modification. The performance of PLA can be enhanced by the incorporation of nanosized particles. Among the nano-sized inorganic particles that are added to the polymer matrix montmorillonite clay are of particular interest due to their abundance, low cost and their geometrical features. The main objectives of this study was to investigate the effect of gamma irradiation on mechanical properties of the poly(Lactic acid)/organoclay nanocomposite films prepared by solution casting method. Also Degradation rate of the PLA samples was investigated by enzymatic degradation test.

2. EXPERIMENTAL

2.1. Materials
PLA granules were purchased from a chemical company in China. Nanosized organoclay with the trade name of Nanolin DK4 (alkyl ammonium modified montmorillonite) with size of 1-100nm and aspect ratio of 100-1000 supplied from Zhejiang clay chemicals, China.
2.2. Preparation of PLA and PLA/organoclay nanocomposites
PLA and PLA/Nanolin DK4 nanocomposites were made via solution casting method [7]. Nanocomposite films were made by addition of Nanolin to the PLA solution. Initially a dispersion of organoclay in chloroform was obtained by introducing various amounts of Nanolin to solvent followed by vigorous mixing in a digital ultrasonic bath for 60 minutes. The solution of PLA and the suspension of Nanolin in chloroform was then mixed together by an ultrasound bath for about 3 hours and poured into a glass mould on a leveled surface. Nanocomposites films containing 2, 4 and 6 pph (pph stands for parts per hundred of the PLA polymer) were made by this procedure.

2.3. Irradiation of films
The pristine PLA and nanocomposite films were cut into strips and irradiated with gamma rays at dose of 30 kGy in presence of air. The irradiation was carried out at room temperature and dose rate of 4.6 Gy/s using a Co60 gamma irradiator facility.

2.4. Characterization of the PLA films
Number average of 100790 g/mol and weight average of 237560 g/mol were obtained for the PLA granule via Gel Permeation Chromatography (GPC) on an Agilent 1100 GPC at temperature of 30°C and solvent of chloroform. The detector was IR (Refractive Index). Chemical structure of the PLA films was studied on Bruker IFS 45 FTIR spectrometer. Gel fraction of the irradiated PLA films was determined using Soxhlet extraction method by following equation.

\[
\text{Gel fraction (\%)} = \left( \frac{W_f}{W_0} \right) \times 100
\]

where \(W_0\) is the initial weight of the irradiated PLA film and \(W_f\) is the weight remaining after 24 h extraction in chloroform followed by drying in a vacuum oven for 48 h.

Mechanical properties of the PLA and PLA nanocomposite films were characterized by a Zwick tensile testing machine. The films were cut into the strips with the dimensions of 10 cm length × 2 cm width and stretched with the gauge length of 4 cm and a speed of 5 mm / min. Thermal behavior and crystalline structure of the samples was investigated via Differential Scanning Calorimetry by the PerkinElmer DSC Model Pyris 1. The test was performed under Nitrogen atmosphere between 40°C and 200 °C at heating rate of 10°C/min.

The structure of the PLA-Nanolin nanocomposite films were evaluated by XRD measurements. Transmission Electron Microscopy was performed on a Phillips EM 208 S electron microscope operating at acceleration voltage of 100 kV. Weight loss studies of the PLA/organoclay nanocomposites and neat PLA films were carried out utilizing enzymatic degradation test. The samples with specified dimensions were placed in vials containing 5 ml Tris Hcl buffer (pH 8.6), 1 mg proteinase K and 1 mg of sodium azide. Then the samples was removed after specified incubation times, washed thoroughly and dried in an vacuum oven for 48 h. Afterward the dried samples were weighed to evaluate weight loss values.

3. RESULTS AND DISCUSSION

3.1. FTIR spectroscopy
No significant changes are observed in the chemical structure of the γ-ray treated sample compared to that of un-exposed one. The FTIR spectra of the PLA /Nanolin nanocomposites (Fig. 1) show the characteristic peaks of the PLA and organically modified nano clay as well. The bands at 516 and
462 cm\(^{-1}\) are assigned to Si-O-Al and Si-O-Si bending vibration of organoclay and strong peaks at 1757 and 1087 cm\(^{-1}\) are corresponding to stretching vibration of C=O and –C–O groups and peak at 1458 cm\(^{-1}\) is assigned to bending vibration of –CH\(_3\) in PLA. The omitting or low intensity of the peak at 800 cm\(^{-1}\) in non-irradiated nanocomposite may be attributed to some interaction between PLA and organically modified clay.

**FIG. 1.** (E) FTIR spectra of (a) neat PLA (b) non-irradiated PLA/organoclay (4pph) (C) irradiated at 30 kGy (F) spectra of (a) PLA/organoclay (4pph) irradiated at 30 kGy (b) the non-irradiated nanocomposite (c) neat PLA.

### 3.2. Mechanical properties

*Gamma rays* are known to induce structural changes, such as scission and crosslinking in the exposed polymers. The change in mechanical properties of the irradiated PLA film implies some structural changes having occurred in the pristine PLA films after exposure to the high energy ionizing radiation. Tensile strength declined markedly from 43.1 to 31.4 MPa and the elongation at break reduced from 10.8% to 9% probably due to some chain scission. Addition of the TAC at 1 wt% improved the tensile strength of the irradiated PLA film but no remarkable change appeared at 2 percent. The gel content of the PLA film containing 1% TAC reduced from 77% to 55.5% upon increasing of TAC to 2 wt%. This suggests the optimum TAC value for crosslink formation between the PLA macromolecules at dose of 30 kGy is 1 wt%.

Incorporating 4 pph of Nanolin further increased the elongation at break of the PLA film to the value of 33.4% and the tensile strength to the value of 31.1 MPa. (Fig. 2) In spite of the lower tensile strength of the 4 pph nanocomposites than that of the neat PLA, the elongation at break increased to as much as three folds. As a matter of fact formation of nanocomposites through introducing layered silicate nanopowder in the PLA changed its structure from a stiff-brittle to a ductile-flexible one. It should be noted that non-irradiated PLA nanocomposites particularly those containing 2 and 4 pph organoclay showed a yield point in their stress-strain curves. Obvious stress whitening and necking were also observed before rupture especially in the 4 pph nanocomposites. Indeed the good dispersion of the clay platelets into the PLA matrix in an exfoliated system and intensive interaction between the two phases is the reason for ductility enhancement of this nanocomposite sample. Increasing the Nanolin content to 6 pph had an adverse effect on the ductility which may be attributed to agglomeration and non-homogenous distribution of the organoclay platelets.
FIG. 2. Ultimate tensile strength and elongation at break of non-irradiated PLA and PLA/Nanolin nanocomposites

The similar trend was observed in the mechanical properties of the PLA nanocomposite films having exposed to 30 kGy gamma-rays except the 2 pph nanocomposite which showed some increase in ultimate tensile strength and reduction in the elongation at break compared to those of the pure PLA. All the irradiated nanocomposite samples exhibited lower elongation at break compared to those of the un-irradiated ones suggesting some chain scission have taken place in amorphous phase of the polymer matrix.

3.4. Morphology

Morphology of the PLA nanocomposites was characterized by X-ray diffraction method and transition electron microscopy.

3.5. X-ray diffraction patterns

Fig. 3 display XRD patterns of the PLA nanocomposites film. The strong sharp peaks in diffractogram of nanocomposites seen at a larger d-spacing and a lower value of 2θ compared to XRD peaks of the Nanolin indicates formation of an intercalated structure, that is insertion of poly(lactic acid) into the silicate layer spaces without disruption of its arrangement. On the other hand the diffraction peak at 2θ=2.38° in the 4 pph nanocomposites has been nearly disappeared (very low intensity of peak comparing to the sharp peak at 2θ = 2.64 in the XRD pattern of the layered silicate) which may be evidence of formation of partially exfoliated nanocomposite at this composition. This can also be observed by the TEM micrographs (Fig.4) of the 4 pph nanocomposites which show fully separated silicate layers randomly dispersed in the polymer matrix.
FIG. 3. X-ray diffraction patterns of PLA-Nanolin nanocomposites at 2θ≤5. The first number on the peak exhibits 2θ° and the second one, d (Å), is the value of the spacing between silicate layers.

3.6. TEM
Figure 4 shows TEM micrograph of the PLA nanocomposite containing 4 pph of Nanolin. As it is seen most of the platelets seem to be well dispersed in a random manner which exhibits exfoliation of layered silicate by the PLA.

FIG. 4. TEM images of (a, b) exfoliated layered silicate in the PLA nanocomposite containing 4 pph Nanolin at different magnifications.

In contrast with the 4 pph, the TEM of the 6 pph nanocomposites (Fig. 5) exhibit an intercalated morphology which shows the individual silicate layers are separated in an organized manner within polymer matrix by an average distance that depends on the clay loading.
3.7. Crystallinity of the PLA nanocomposite

Crystallinity of the PLA nanocomposites was investigated by differential scanning calorimetry (DSC). Fig. 6 display DSC thermograms of the neat non-irradiated and irradiated PLA films. An endothermic peak at temperature at 149°C is observed which represents melting point of the PLA and the other endothermic peak at temperature of around 53°C corresponds to the glass transition of the PLA. All the nanocomposites containing 2, 4 and 6 pph of layered silicate show higher enthalpy and temperature of melting compared to those of the pure PLA indicating growth of crystalline region in the nanocomposite structure (Table.1). Incorporation of modified clay enhances crystallites growth as the nanosized particles act as nucleating sites. Also the enthalpy of melting of the irradiated neat PLA has increased significantly which may be attributed to the increasing of crystallinity upon exposing it to 30 kGy of γ-irradiation dose. Indeed interaction of high energy radiation with the sample leads to chain scission and consequent shorter chains which facilitate creation of crystallites with smaller size (Fig.6)
TABLE 1. THERMAL BEHAVIOR OF NEAT PLA AND PLA NANOCOMPOSITES CONTAINING 2,4,6 PPH LAYERED

<table>
<thead>
<tr>
<th>Sample code</th>
<th>$T_g$ (°C)</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_m$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA0</td>
<td>53</td>
<td>149.22</td>
<td>13.56</td>
</tr>
<tr>
<td>PLA30</td>
<td>53.52</td>
<td>149.77</td>
<td>29.67</td>
</tr>
<tr>
<td>2pph</td>
<td>70.75</td>
<td>151.33</td>
<td>26.88</td>
</tr>
<tr>
<td>4pph</td>
<td>71</td>
<td>150.74</td>
<td>24.30</td>
</tr>
<tr>
<td>6pph</td>
<td>71</td>
<td>150.45</td>
<td>20.68</td>
</tr>
</tbody>
</table>

The glass transition of the nanocomposites also indicate a significant increase over $T_g$ of the pristine PLA film implying strong interaction of organically modified silicate layers with polymer matrix which retards movement of the polymer chain segments.

3.8. Enzymatic degradation test

Fig.7 shows the weight loss of neat PLA and nanocomposite films containing 2 and 4 pph organically modified layered silicate at 0 and 30 kGy $\gamma$ irradiation dose. As seen in the Fig. 7 all the films nearly degrade with the same moderate speed except the neat irradiated PLA film which shows almost no weight loss after 48 h and then its degradation rate increases rapidly. Interestingly the weight loss in both irradiated and non-irradiated nanocomposites containing 2 pph organoclay is also slower than other samples. This is implying that diffusing enzyme in the crystalline region is harder than that of amorphous region as verified by higher enthalpy of melting in these samples compared to that of pure PLA. The PLA weight loss is nearly similar to the non-irradiated nanocomposite with 4 pph organoclay. This can be explained by exfoliated morphology of this nanocomposite and randomly dispersion of layered silicate in the PLA matrix which makes it easier for enzyme to diffuse compared to the samples with intercalated structure. Irradiated 4pph nanocomposite shows more weight loss because of some chain scission induced by gamma irradiation. Apparently the rate of weight loss depends on both morphology and degree of crystallinity of the sample.

**FIG. 7.** Weight loss of the neat PLA films and nanocomposites containing 2 and 4 pph organically modified layered silicate at 0 and 30 kGy $\gamma$ irradiation dose.
3.9. Preliminary results on PLA/Hydroxapatite nanocomposites

The preliminary results on mechanical properties of the PLA/Hydroxapatite (HAP) nanocomposites containing 10 and 20 pph of HAP showed the increase of ductility particularly at 10 pph (Fig.8). Irradiation of these samples at dose of 30 kGy did not deteriorate the mechanical properties of the nanocomposites as the tensile strength remained nearly constant and elongation at break increased significantly at the sample containing 10 pph of HAP. Further investigation is needed to obtain PLA/HAP nanocomposite systems with desired properties.

**FIG. 8.** (a) Ultimate tensile strength and elongation at break of non-irradiated PLA and PLA/HAp nanocomposites at 0 kGy (b) at 30 kGy.

4. CONCLUSIONS

Nanocomposites of the poly (Lactic acid)/layered silicate were successfully prepared via solution casting method. Irradiation of pristine poly (Lactic acid) films with gamma rays at 30 kGy deteriorated the mechanical properties of the exposed films. Addition of TAC monomer at 1 wt% slightly increased the tensile strength and decreased elongation at break suggesting three dimensional network formations upon exposure to high energy radiation at 30 kGy. Introducing Nanolin DK4 into the poly (Lactic acid) films significantly improved the elongation at break of the hybrid samples particularly in the 4 pph nanocomposite. Exposing the PLA nanocomposites to 30 kGy gamma rays led to the slightly lower ultimate tensile strength and lower elongation at break probably due to some chain scission. Formation of the nanocomposites was verified by TEM images and XRD patterns. Incorporation of the nanosized organophilic clay enhanced crystallites growth in the nanocomposite samples due to the action of the nanosized particles as nucleating sites. The enzymatic degradation rate of the PLA film changed depending on both the morphology and crystallinity of the samples. Mechanical properties of the PLA/HAP was dependent on the composition and morphology of the prepared samples. The ductility of the irradiated PLA nanocomposites containing 10 pph of HAP increased 3 times compared to that of the neat irradiated PLA.

ACKNOWLEDGMENTS

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REFERENCES


RADIATION ENGINEERED NANOGELS AS PLATFORM FOR NEXT-GENERATION OF MEDICAL DIAGNOSTICS AND THERAPEUTICS

C. Dispenza, M.A. Sabatino, S. Alessi, G. Spadaro; ITALY

1. INTRODUCTION

Nanotechnology holds the promise of enabling new materials and systems to accomplish delicate and demanding roles in healthcare for medical diagnostics (imaging and sensing) and therapeutics (drug delivery). The development of functional nanoparticles that can ensure high MR imaging contrast and/or molecular recognition functions; or specific medical intervention at the molecular scale for treating a disease or repairing damaged tissues are currently at the agenda of many public and private research institutions and agencies.

Several different nanomaterial systems have been proposed for application in nanomedicine: including colloidal systems (such as liposomes or microemulsions) [1], quantum dots (QDs) [2], organic and inorganic dendrimers [3], viral and virus-like nanoparticles [4], polymeric vesicles and solid lipid nanoparticles [5]. Each of these systems has advantages and disadvantages, often related to in vivo toxic side-effects or difficult scalability of their production process at industrial level. Nanogels, or small particles formed by physically or chemically crosslinked polymer networks, represent a niche in the development of “smart” nanoparticles for drug delivery and diagnostics [6]. They offer unique advantages over other systems, including a large and flexible surface for multivalent bio-conjugation; an internal 3D aqueous environment for incorporation of large (bio)molecular drugs, the possibility to entrap active metal or mineral cores for imaging or phototherapeutic purposes; stimuli-responsiveness and biocompatibility. Moreover, conformability and flexibility make these nanoparticles able to penetrate through small pores and channels through shape modification. Major synthetic strategies for the preparation of nanogels belong to either micro-fabrication methodologies (photolithography, microfluidic, micromoulding) or to self-assembly approaches that exploit ionic, hydrophobic or covalent interactions [7]. For the latter, in particular, dimensional control has been often achieved by the recourse to soft templates, such as the aqueous droplets dispersed in organic solvents of heterogeneous colloidal systems. While micro-fabrication methods are limited by the need for the use of costly equipments, the recourse to surfactants as well as organic solvent, initiators and catalysts may detrimentally affect the toxicological profile of the nanogels produced in colloidal systems. The availability of inexpensive and robust preparation methodologies is at the basis of the development of effective nanogel-based theragnostic devices.

The design of functional nanoparticles must include a high degree of control of product properties through both process and material chemistry. There are a few basic important criteria that need to be carefully considered in designing such nanodevices. One main issue is related to the hydrodynamic size and shape of nanocarriers. Further requirements in design include eventual protection of the payload from degradation by cell’s metabolic system as well as control of the pharmacokinetics, sterility and absence of cytotoxicity and appropriate removal post-usage strategies of the devices.

High energy radiation processing demonstrated its potential for the production of nanogels already in the late ‘90s, owing to the pioneeristic work of Rosiak and collaborators, but since no adequate efforts have been spent in developing a viable technology based on this ground to develop radiation
engineered multi-functional nanoparticles with the required properties, at a preference to other approaches [8]. The aim of the present work is to assess the possibility of generating biocompatible multifunctional nanogels with dimensions varying in a range of tens to hundreds of nanometers using existing industrial-type accelerators and set-ups. In the further development of the research the functional groups grafted to the networks will be used to attach selected targeting ligands. Furthermore, GdIII-complexes will be incorporated as contrast agents to enable in vivo biodistribution studies through NMR imaging.

2. EXPERIMENTAL

2.1. Materials

PVP k60 (Aldrich, 

M_w=1,60x10^5 g/mol), fluorescein diacrylate, (FDA, Aldrich), (3-amino propyl)methacrylamide hydrochloride (APMAM, Polyscience), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ortho-phosphoric acid, hydrochloric acid (37%), sodium hydroxide, sodium chloride, have been used as received without further purification. The PVP molecular weight, 

M_w = 4,1 x10^5 g/mol and the radius of gyration, R_g = 27 nm, were estimated from the usual Zimm plot analysis of static light scattering measurements carried out at 25 °C.

2.2. Sample preparation

PVP aqueous solutions at 0.25 and 0.1 wt% were prepared by overnight stirring and filtered with 0.22µm pore size syringe filter (Millipore), carefully deoxygenated with gaseous nitrogen and bottled in 15 ml glass vials sealed with rubber septa and aluminum caps. PVP aqueous solutions at 0.1 wt% were also formulated with FDA and APMAM at two different concentrations. Samples were individually saturated with N_2O prior to irradiation in order to increase the concentration of hydroxyl radicals formed from water radiolysis during irradiation.

Electron beam irradiation was performed using two different 10 MeV liner accelerators LAE 13/9 and Electronika 10/10 at the Institute of Nuclear Chemistry and Technology of Warsaw (Poland). Irradiations conditions for LAE 13/9 were varied by varying the average current and the irradiation time. Three sample vials per run were placed horizontally in front of the electron emission port in a suitable container filled with ice. Irradiation with Electronika 10/10 was carried out at the following doses: 40, 80kGy. The total doses were obtained by multipass exposure (ca. 40 kGy per one pass). Also in this case samples were horizontally placed in a box filled with ice. Samples are conveyed under the beam via a transporting belt, therefore the number of vials irradiated per run were of several tens. After irradiation samples were dialyzed against distilled water for 48 h using dialysis tubes of 12,000 Da cut-off (Aldrich) to remove low molecular weight residues.

2.3. Characterizations

An estimation of the yield of the process was determined gravimetrically by comparing the dry weight of the polymer in the sample before and after irradiation followed by dialysis and freeze-drying.

The R_g and R_h (gyration and hydrodynamic radius) of the not irradiated PVP samples were measured by static and dynamic light scattering (DLS) techniques, respectively, using a Brookhaven Instruments BI200-SM goniometer. Samples were put in a thermostated cell
compartment of the instrument and temperature was controlled to within 0.1°C using a thermostated recirculation bath. The light scattered intensity and time autocorrelation function were measured by using a Brookhaven BI-9000 correlator and a 100 mW Ar laser (Melles Griot) tuned at \( \lambda = 632.8 \) nm. Measurements were taken at different scattering vector \( q = 4\pi n\lambda_0^{-1} \sin(\theta/2) \) where \( n \) is the refraction index of the solution, \( \lambda_0 \) is the wavelength of the incident light, and \( \theta \) is the scattering angle. Static light scattering data were corrected for the background scattering of the solvent and normalized by using toluene as calibration liquid. In dynamic light scattering experiments the correlator was operated in the multi-\( \tau \) mode; the experimental duration was set in order to have at least 2000 counting on the last channel of the correlation function. Samples were placed in the quartz cell used for the measurement, as produced, after dilution with bidistilled water and/or syringe filtration (5, 1.2, 0.8, 0.45 and 0.22 \( \mu m \) pore size - Millipore). Reported curves are representative of the behavior of different preparation for each formulation.

FTIR analysis was carried out using a Perkin Elmer spectrophotometer. Spectra were recorded at 30 scans per spectrum, 1 cm\(^{-1}\) resolution on freeze-dried samples dispersed in KBr and compressed into pellets.

Surface morphology was imaged by a field emission scanning electron microscopy (FESEM) system (JEOL) at an accelerating voltage of 10 kV. Samples for FESEM were coated with a gold layer by JFC-1300 gold coater (JEOL) for 30 s at 30 mA before scanning.

Zeta potential of the nanogels was detected by the laser Doppler anemometry (Zeta Plus Analyzer, Brookhaven Corporation, USA).

3. RESULTS AND DISCUSSION

The yield of the process in terms of purified dry product for all formulations and irradiation conditions is always close to 100 %. This indicates that irradiation in the specified conditions is not inducing chain-scission to an appreciable extent.

In the view of the envisaged application of the radiation engineered nanogels as nanocarriers for injectable drug formulations an initial screening of irradiation conditions was carried out on the basis of dynamic light scattering measurements of the hydrodynamic diameter of the generated nanogels.

**Base PVP nanogels**

The commercial grade PVP used is characterized by a fairly wide molecular weight distribution (polydispersity index 2.6). This polymer was preliminarily characterized for its \( R_g \) (gyration radius) and for its \( R_h \) (hydrodynamic radius) in water in the concentration range of 0.05-0.25 wt.%. These values lay below the critical concentration chain “overlap” concentration for the polymer, that was estimated to be approximately 1% [9].

Data in figure 1 are relative to autocorrelation functions, measured at 90° scattering angle, for the not-irradiated and irradiated (40kGy-LAE-24 min) PVP 0.25 % systems, respectively. Similar
results were obtained at different scattering angles. Upon irradiation, the autocorrelation function is observed to decay at longer times. Data were fitted to a double exponential (figure 1-c,d):

$$g^{(2)}(t)^{\ast 2} = [A_0 + A_1 \exp\left(\frac{-t}{\tau_1}\right) + A_2 \exp\left(\frac{-t}{\tau_2}\right)]^{\ast 2}$$

where $A_{i=1,2}$ and $\tau_{i=1,2}$ are the amplitude and decay time. This corresponds to describe the autocorrelation function in terms of two species of different size. The amplitude of each exponential is proportional to the contribution of each species to the scattered intensity, which, in turn, is directly proportional to the weight-averaged molar mass multiplied the square of the number concentration. Assuming for both species a similar dependence of the weight-averaged molar mass and gyration radius, it is also possible to estimate the numerical ratio $N_1/N_2$ between the smaller and the bigger objects. Fitting to a single exponential was considered unsatisfactory. The comparison between the two curves is presented in figure 1-a, while data analysis using a commercial software (CONTIN) gives for both systems the intensity-weighted particle size distributions reported in figure 1-b. From this panel, it can be noticed that the not irradiated PVP at 0.25 wt. % is characterized by a bimodal size distribution of objects in water. Therefore, the smaller average hydrodynamic diameter calculated (see figure 1-c) could be associated to single chain coils, while the bigger may correspond to either small aggregates of few chains through water-mediated hydrogen bonds or to high molecular weight chains. It is worth pointing out that the same description holds for PVP aqueous solutions at the lower concentration (0.1 wt.%).

Irradiation at 40 kGy and at the lowest “average” dose rate induces an increase of both the two characteristic dimensions, with a more pronounced effect on the bigger. The comparison between the not irradiated and the irradiated system suggests that irradiation in these conditions is inducing both inter- and intra-molecular crosslinking, even if the polymer concentration is lower than the concentration of overlap. Owing to the relatively short inter-radical distance between macroradicals formed in different chains, their intermolecular recombination is a possible occurrence.
Dilution of the irradiated systems and filtration with 220 nm pore size filters, figure 2 (a-b), is not showing appreciable effects in the decay times of the autocorrelation functions, thus proving that the objects generated upon irradiation are dimensionally “stable”.

FIG. 1. DLS analysis of PVP at 0.25 % in water before and after E-Beam irradiation
The influence of the irradiation conditions and, in particular, of the average electron beam current intensity on irradiated PVP solutions at 0.25 wt.% in terms of average diameters of the two distributions is shown in table 1. When the higher irradiation dose is delivered in a shorter length of time smaller nanoparticles are obtained, as a result of the higher concentration of radicals instantaneously formed on the chains, making their intra-molecular recombination more favorable.

**TABLE 1**

<table>
<thead>
<tr>
<th>PVP 0.25 wt. %</th>
<th>n irr.</th>
<th>40kGy LAE 24 min, 80kGy LAE 10 min,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1 (nm)</td>
<td>D2 (nm)</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>44</td>
</tr>
</tbody>
</table>

At a lower concentration of polymer in water the average distance between macromolecules further increases and intra-molecular recombination of radicals prevails. Hydrodynamic diameters for the PVP 0.1 wt.% irradiated solution from the fitting are reported in table 2. For these systems the
difference with the corresponding not-irradiated system is less marked than for PVP 0.25 wt.%. In particular, the system irradiated at the lowest dose and lowest average dose rate shows only slightly higher diameters, while for the highest dose and higher dose rate decay times are shorter than those of the linear polymer. In these conditions, irradiation is causing a rapid and significant shrinkage of the polymer coils. Irradiation at 80 kGy, carried out with two passes at the highest dose rate (with Elektronika), is leading to comparable results.

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>n. irr.</th>
<th>40kGy LAE 24 min</th>
<th>80kGy LAE 10 min</th>
<th>80kGy ELEKT</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 (nm)</td>
<td>13</td>
<td>14</td>
<td>25*</td>
<td>24</td>
</tr>
<tr>
<td>D2 (nm)</td>
<td>53</td>
<td>78</td>
<td>--</td>
<td>384</td>
</tr>
<tr>
<td>N1/N2</td>
<td>163</td>
<td>2,245</td>
<td>--</td>
<td>1,600,000</td>
</tr>
</tbody>
</table>

*Fitting with a single exponential

FTIR analysis has been carried out on the freeze-dried nanogels in order to estimate the extent of any eventual chemical modification of PVP structure upon irradiation in the different conditions.

![FTIR spectra of not irradiated PVP and PVP nanogels at 80 kGy.](image)

**FIG. 3.** FTIR spectra of not irradiated PVP and PVP nanogels at 80 kGy.

Figure 3 shows the comparison between the spectra of not irradiated PVP and PVP 0.1% irradiated at 80 kGy for 10 min in the range 2000-400 cm\(^{-1}\). All the bands characteristic of the linear PVP are present in the spectrum of the nanogel and two new peaks at 1772 and 1703 cm\(^{-1}\), falling in the envelope of the prominent PVP carbonyl band. These peaks can be associated to 5 membered cyclic imides. A possible explanation of this modification is proposed in the scheme 1. Confirmation of the proposed structure will be sought in solid-state \(^{13}\)C\(^{1}\)H-NMR spectra.
Scheme 1: Structures of polymer radicals formed upon irradiation and possible follow-up reactions.

*Functionalised PVP nanogels*
Fluorescein diacrylate (FDA) or (3-aminopropyl)methacrylamide hydrochloride at two concentrations were added to the PVP aqueous solution at 0.1 wt.% and irradiated at 80 kGy, in order to provide light emissive properties for biological evaluation through confocal microscopy studies and primary amino groups for subsequent bioconjugation with targeting ligands.

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>PVP 0.1% 80 kGy LAE 10 min</th>
<th>+FDA</th>
<th>+ APMAM (+)</th>
<th>+ APMAM (++)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D1 (nm)</strong></td>
<td>25*</td>
<td>25</td>
<td>243</td>
<td>259</td>
</tr>
<tr>
<td><strong>D2 (nm)</strong></td>
<td>--</td>
<td>458</td>
<td>3,795</td>
<td>4,870</td>
</tr>
<tr>
<td><strong>N1/N2</strong></td>
<td>--</td>
<td>1,250,000</td>
<td>36,020</td>
<td>83,000</td>
</tr>
</tbody>
</table>

*Fitting with a single exponential

**TABLE 4**

<table>
<thead>
<tr>
<th></th>
<th>+ APMAM (++)</th>
<th>pH 4</th>
<th>pH 6.8</th>
<th>pH 7.4</th>
<th>pH 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D1 (nm)</strong></td>
<td>144</td>
<td>300</td>
<td>306</td>
<td>131</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 reports the calculated hydrodynamic diameters for the functionalized nanogels. The presence of FDA does not affect the dimensions of the main population of particles (25 nm), while APMAM increases this dimension of one order of magnitude (~250 nm). The nanoparticles acquire pH responsiveness in virtue of the primary amino groups grafted, as shown in table 4.

In vitro biocompatibility and immunolocalisation studies (data not reported) are indicating total absence of toxicity and the ability of the generated functional nanoparticles to bypass the cell membrane of cultured osteoblasts.
4. CONCLUSIONS

The research activity carried out during the first term of the CRP has established that crosslinked nanogels can be produced using industrial type accelerators. Control of the particles dimensions through the extent and proportion between inter- and intra-molecular crosslinking can be achieved varying the polymer concentration in water and the irradiation conditions. The yield of the process in terms of recovered product after purification is close to 100%. Irreversible (covalent) attachment of a fluorescent dye and of a functional (primary amino group carrying) monomer can be also obtained. The functional nanoparticles generated are dimensionally stable, redispersible from the freeze-dried solid state, pH responsive and not-cytotoxic.

REFERENCES

INTRODUCTION OF FUNCTIONAL STRUCTURES IN NANO-SCALES INTO ENGINEERING POLYMER FILMS USING RADIATION TECHNIQUE

Y. Maekawa; JAPAN

Summary

Introduction of functional regions in nanometer scale in polymeric films using γ-rays, EB, and ion beams are proposed. Two approaches to build nano-scale functional domains in polymer substrates are proposed: 1) Radiation-induced grafting to transfer nano-scale polymer crystalline structures (morphology), acting as a nano-template, to nano-scale graft polymer regions. The obtained polymers with nano structures can be applied to high performance polymer membranes. 2) Fabrication of nanopores and functional domains in engineering plastic films using ion beams, which deposit the energy in very narrow region of polymer films. Hydrophilic grafting polymers are introduced into hydrophobic fluorinated polymers, cross-linked PTFE (cPTFE) and aromatic hydrocarbon polymer, poly(ether ether ketone) (PEEK), which is known to have lamella and crystallite in the polymer films. Then, the hierarchical structures of graft domains are analyzed by a small angle neutron scattering (SANS) experiment. From these analyses, the different structures and the different formation of graft domains were observed in fluorinated and hydrocarbon polymer substrates. The obtained polymers with nano structures can be applied to high performance polymer membranes. For nano-fabrication of polymer films using heavy ion beams, the energy distribution in radial direction, which is perpendicular to ion trajectory, is mainly concerned. For penumbra, we re-estimated effective radius of penumbra, in which radiation induced grafting took place, for several different ion beams. We observed the different diameters of the ion channels consisting of graft polymers. The channel sizes were quite in good agreement with the effective penumbra which possess the absorption doses more than 1 kGy.

1. INTRODUCTION

We have been attempting two approaches to introduce functional regions in nanometer scale in polymeric films using γ-rays, EB, and ion beams are proposed: 1) Radiation-induced grafting to transfer nano-scale polymer crystalline structures (morphology), acting as a nano-template, to nano-scale graft polymer regions. The obtained polymers with nano structures can be applied to high performance polymer membranes. 2) Fabrication of nanopores and functional domains in engineering plastic films using ion beams, which deposit the energy in very narrow region of polymer films.

As shown in Fig. 1 (a), one of the advantage EB and γ-rays should be high transmittance property. Thus, polymer films can be received the energy homogeneously along the film thickness direction. However, most of polymer films have crystallites in nano-scale. Since active species (radicals) generate only in a crystalline region, the functional domains are propagated from the nano scale crystalline templates when functional monomers was added [1]. The hybrid polymer films can be also utilized; namely, inorganic crystals should act as a template for the new functional regions. In this case, we can change the size and shapes of the crystallites.
We have recently applied this technique to the development of polymer electrolyte membranes for fuel cells. Fuel cells show high power generation efficiency, so it has been expected to solve energy

FIG. 1. Two approaches to build nano-scale functional domains in polymer substrates (a) Radiation-induced grafting to transfer nano-scale polymer crystalline structures acting as a nano-template to nano-scale graft polymer regions. (b) Fabrication of nanopores and functional domains in engineering plastic films using ion beams, which deposit the energy in very narrow region of polymer films.
resource depletion. Especially for hydrogen type fuel cells, such as residential co-generation system, and FCHV, fuel cell vehicles, should reduce CO2 emissions, which is thought to be the main reason for green house effects. Hydrogen and methanol can be used as a fuel. We have been developing both direct methanol and hydrogen fuel types. DMFC has been developed for very compact mobile type devices such as mobile phone and note PC. The main goals of hydrogen fuel cells are for residential co-generation system and Fuel cell vehicles. The natural gas converts to H2 and by fuel cell electricity are generated. At the same time, the fuel cell generates the heat. By using heat, we make hot water to supply it to bath or kitchen to achieve more than 70 % energy efficiency. And this is a fuel cell vehicles, Energy efficiency is more than 50 % and is much higher than engine type automobile. And also it emits only water and is very nice for environment because of reduction of CO2 emission. Of course the FCHV is expected to have very large market size.

When polymeric films are irradiated, radials are generated in the films. Then, if two generated radicals react with each other, cross-linking is incorporated, which can enhance the mechanical strength of polymer films. On the other hand, if functional monomers exist in the system, graft polymerization proceeded to introduce functional graft polymers in the films. Another advantage of the radiation technique is to achieve the required properties of PEM by introducing functional polymer grafts and selected polymer film substrates. By choosing them or combination of these two components, the required properties of PEM have been improved [2].

The second approach is the nano-fabrication of engineering plastic films using ion beams [3]. One ion just deposits its energy into one nano to micro-meter scales in the polymer films. Then, nanopores can be generated by resolving the damaged area or functional regions can be introduced by grafting functional monomers into the area. Compared with other energetic particles, heavy ion beams give very narrow nanoscale energy deposition through straight ion trajectory. The trajectory of the heavy ion beam, Xe ions with 450 MeV incident energy is amazingly narrow with very little broadening to be a few nano meter in more than 40 µm depth of the polymer films. In other words, high energy heavy ion beam is the very interesting tool to give nano structures with very high aspect ratio in polymer film with 10 to several hundred µm thickness.

When one ion particle penetrates into polymer films or any materials, the energy was deposited to polymer films with the distribution of the film thickness direction. Furthermore, the any position of ion trajectory in the film has another energy distribution along radial direction, namely, perpendicular to the direction of ion beam penetrating. Thus, one can obtain three dimensional energy distribution in polymer films and these distribution can be controlled by changing mass and acceleration energy of ion beam.
First, fluorinated polymers, cross-linked polytetrafluoroethane (cPTFE) and poly(ethylene-co-tetrafluoroethylene) (ETFE) were irradiated with γ-rays. Then, the films were immersed in styrene solutions to introduce polystyrene grafting chains. Subsequently, the obtained grafting chains are sulfonated with chlorosulfonic acid in dichloroethane to give grafted type fluorinated polymer electrolyte membranes. At the first stage of our research, we had develop direct methanol type PEM using the technique suing the above fluorinated polymers [2,4]. Since PEM for hydrogen type FC requires higher thermal durability and mechanical strength, aromatic hydrocarbon polymers were employed. Aromatic hydrocarbon polymer, poly(ether ether ketone) (PEEK), which is one of the most highly performance aromatic hydrocarbon polymers. As expected, PEEK films are very thermally and chemically stable; so far, there had been no report about successful graft polymerization. Grafting speed was very slow and after 3days only 30% GD can be obtained. However, PEEK films originally possess stable radicals and thus, thermal grafting of divinylbenzene (DVB) proceeded. Namely, first, DVB was introduced into PEEK films as a scaffold of radiation-grafting. Then, we radiation grafting of sulfo group containing monomer could be grafted into PEEK/DVB membranes. With only hydrolysis in water at 95C, it could be converted to PEEK-based PEM with ion conductivity of more than 0.11 S/cm, which is 1.5 times higher than Nafion, and 2.3 times higher mechanical strength compared with Nafion [5].
By using the membrane electrode assembly (MEA) using the prepared PEEK-based PEM, fuel cell performance was tested. Under the fuel cell operation test at 95 °C under 80% RH with 300 mA, this membrane kept cell voltage at least 1000 hours much more stable than Nafion under the same condition. From the acceleration test, the lifetime under 80°C was estimated to be as comparable to 40,000 hours, which meets the target of residential co-generation system.

The structures of the graft-type PEM consisting of fluoro and hydrocarbon polymer substrates were characterized in nano- to meso-scale using small-angle neutron scattering (SANS). Fig. 4 showed the SANS profile of cPTFE, grafted cPTFE, and cPTFE-base PEM and proposed structures of the PEM in nano- to meso-scales. The clear peak at the position of correlation length of 45 nm was observed in the profile of cPTFE substrate, which should correspond to the correlation distances between crystallites. The SANS profile of the grafted cPTFE has a similar profile to the cPTFE substrate, in which the peak for the correlation length of 47 nm appeared with relatively higher intensities in the Q region, compared with that of the cPTFE substrate. Namely, the grafting layers should propagate under the influence of the crystallites. A slight increase of the correlation distance from 45 to 47 nm should correspond to expand of the films owing to the introduced graft polymer layers on the crystallites. Since the profile of cPTFE-based PEM is almost the same as that of the precursor grafted cPTFE, the sulfonation reaction of polystyrene grafts did not affect the size and shape of the grafted cPTFE film. The grafted cPTFE and cPTFE-base PEM exhibited $Q^2$ power law behavior, which is well explained by Porod’s Law as being due to the sharp interface between the graft domains and the FEP substrates. This is because polystyrene and poly(styrenesulfonic acid) grafts were not miscible to the fully fluorinated cPTFE substrate.
Fig. 5 shows SANS profiles of PEEK, grafted PEEK, and PEEK-base PEM and proposed structures of the PEM in nano- to meso-scales. SANS profiles of the original PEEK substrate showed no clear scattering while the grafted PEEK exhibited much stronger scattering over the whole Q range and a new shoulder-like peak at d-spacing of 13 nm. The SANS profile of the PEEK-PEM had a similar profile with a shoulder-like peak at d-spacing of 12.6 nm. The decrease in d-spacing was in good agreement with the decrease of the volume of the grafts due to the elimination of the ethyl group by hydrolysis. The above SANS results clearly show that the size of ion channels (13 nm) formed by PSSA grafts is 2.5 times larger than that in Nafion.

The asymptotic Q-behavior of $Q^{-2}$ at the higher Q-region of d-spacing of 13 nm indicates that there was no sharp interface between the grafts and PEEK substrate. Since both cPTFE and PEEK based grafted films possess the polystyrene derivatives consisting of only hydrocarbons but not fluorine atoms, the different interfacial structures, clear and unclear boundaries, should result from different solubility of the graft polymers with perfluorinated or aromatic hydrocarbon polymer substrates; namely, the graft polymers are miscible with the PEEK chains but not miscible with the cPTFE chains. From these analyses, the different structures and the different formation of graft domains were observed in fluorinated and hydrocarbon polymer substrates. In the case of cPTFE, the grafted domain, working as an ion channel, grew to cover the crystallite, resulting in the similar domain size to that of the crystallite. On the other hand, the PEEK PEM has smaller domain size and it seems to grow independently on the crystallite of PEEK substrate [6].
3. NANO-FABRICATION OF POLYMER FILMS USING ION BEAMS

**FIG. 5.** SANS profiles of PEEK, grafted PEEK, and PEEK-base PEM and proposed structures of the PEM in nano- to meso-scales.

**FIG. 6.** Schematic structures of the grafted PEM prepared from fluorinated polymer substrate, cPTFE and aromatic hydrocarbon polymer, PEEK.
For nano-fabrication of polymer films using heavy ion beams, we took notice of energy distribution in radial direction, which is perpendicular to ion trajectory. The quite high energy is deposited at the center where beam just pass through, called as track core and the deposited energy gradually decreased with increases of radial distance from the center at penumbra area. Track core is defined by physical parameters; the most of the ions with wide range of energy possess the track core radius of about 1nm. Penumbra is defined as the distance which knock-on electron from the core reaches, which in general is several μm.

The energy at radial distance r is expressed by LET, track core radius, and penumbra radius as a parameter [7]. The energy distribution of 450 MeV Xe, irradiated to a Teflon film was re-estimated as shown in Fig. 7. At r = 1nm, the absorbed energy is about 10MGy and about half of the energy deposited in the core. Therefore, when the track core is etched in appropriated etchant, the films with through holes perpendicular to the irradiated films, called ion track membranes, can be produced. The effective radius of penumbra, which we took notice for applications, were re-estimated. Since the graft polymerization to prepare fuel cell membranes, requires 1kGy as a minimum absorbed energy, the effective radius as about 110 nm because the area in the circle of 110 nm radius have the deposited energy of more than 1 kGy. When the ion beams were changed to 450 MeV Fe and 100 MeV oxygen, the effective radius of penumbra decreased from 110 nm to 78 nm and 26 nm, respectively.
In SEM and TEM photographs of the PEM prepared by Xe ion grafting, the straight ion channels in the parallel direction of ion beam trajectory can be observed. The different diameter of the ion channels consisting of graft polymers can be clearly observed. The channel sizes are quite in good agreement with the size of effective penumbra which possess the absorption doses more than 1 kGy.

In the coming year, we continue the both projects. For the nano transfer to polymer films using EB and γ-rays, the shape and size of crystallites in polymer substrates will be controlled. The shapes and sizes of graft domains, which generate from the nano crystallite, will be compared with those of the crystallites. For ion beam nano fabrication, nano pore formation of fluorinated polymers such as PVDF will be examined. These films should be applied for selective separation membranes, reactors including catalysts for pharmaceutical synthesis and DNA recognition for biosensors.

**FIG. 7.** The energy distribution of 450 MeV Xe irradiated to a Teflon film and the re-estimated effective penumbra radius obtained by irradiation of 450 MeV Xe, 400 MeV Fe, and 100 MeV O atoms.
REFERENCES


GAMMA RADIATION INDUCED PREPARATION OF FUNCTIONAL CONDUCTING POLYMER HOLLOW SPHERES

K.-P. Lee, A.I. Gopalan, M.F. Philips, K.M. Jeong; KOREA, REP. OF

1. INTRODUCTION

New materials are sought for applications in many of the emerging fields that include catalysis, sensors, biomedical, optics and electronic application. With the advent of nanotechnology, innovative materials with novel properties are being synthesized towards target applications. Changing the sizes of particles, chemical, optical, and mechanical properties of the materials can often be tailored according to the specific needs of the application [1]. Nanocrystalline, nanoparticles, nanocapsules, nanoporous materials, nanofibers, nanowires, fullerences, nanotubes, nanosprings, nanobelts, dendrimers and nanospheres, etc, are few of the nanostructured materials. The examples of nanostructured materials include semiconducting nanowire quantum dots for gas sensing [2] and self-assembled flower-like architectures [3]. Self-assembly of nanoparticles can result in specific structures with unique and useful electronic, optical, and magnetic properties [4]. Self or induced assembly of simple nanoparticles and rods could result into complex geometries, such as nanoflowers, binary superlattices, optical grating [5,6].

Over the past decade, hollow spherical nanomaterials have received considerable attention due to their interesting properties such as low density, high surface area and good permeation. Various methods like solvothermal, self-assembly, sonochemical, solvent evaporation, chemical vapor deposition, microwave-assisted aqueous hydrothermal and electrochemical are being pursued for the production of hollow spherical materials. Polymer capsules and hollow spheres have increasingly received interest because of their large surface area and potential applications in catalysis, controlled delivery, artificial cells, light fillers and photonics [7].

Recently, conducting polymer (CP) nanostructures such as nanotubes, nanodiscs and hollow spherical nanoshells have received interest because of their physico-chemical properties and potential applications [8]. A variety of synthetic approaches which include interfacial polymerization [9], etc., have been developed for the preparation of CP nanostructures. In recent years, there has been increasing interest in the fabrication of composite hollow spheres consisting of CP as the support matrix and MNPs) or other catalyst particles as the other component [10].

Liao et al [11] reported the preparation of water-dispersible polypyrrole (PPy) nanospheres with diameters of less than 100 nm. A hollow spherical-nanostructured conductive polymer/metal oxide composite has been synthesized [12]. Mallick et al [13] reported the preparation of paramagnetic polyaniline (PANI) nanospheres (PANI–cerium(III) supramolecular composite) by an in situ synthesis. PANI/Fe3O4 composite hollow spheres have been successfully synthesized using sulfonated polystyrene spheres as the template [7]. A novel method has been established for the preparation of CP nano-spheres with controllable sizes [14]. A simple and one-step method has been reported for the fabrication of poly(aniline-copolyrrole) copolymer hollow nanospheres via the oxidative polymerization of a mixture of aniline and pyrrole in the presence of Triton X-100 [15]. Oxidative polymerization of aniline with ferric chloride resulted urchin-like PANI composite hollow spheres [16]. Hollow PANI spheres have been synthesized with latex sphere template [17].
Recently, Wan has developed an “emulsion template” approach to prepare hollow PANI spheres [18]. Li et al [19] reported the preparation of core-shell nanostructured conductive PPy composite. New studies for establishing methods for nanoscale engineering materials, especially towards biomedical applications are warranted.

We report the preparation of hollow spherical nanocapsules (HSNC) and subsequent modification onto hollow spherical composite nanocapsules (HSCNC) by loading MNPs into the HSNC(PPy) through $\gamma$-radiation. The HSCNC is designated as HSCNC(PPy/MNP) in terms of the shell (PPy) and loaded metal nanoparticles (MNPs). The preparation of HSCNC(PPy/MNP) involves steps as detailed in Scheme 1. In this case, $\gamma$-irradiation was used for loading MNPs onto HSNC(PPy). Also, we have utilized the above methodology for the preparation of HSNC based on PANI using $\gamma$-irradiation. We have prepared PANI based HSNC(PANI) and HSCNC(PANI/MNP). HSCNC(PPy/MNP) and HSCNC(PANI/MNP) were independently prepared by different approaches to bring out the effectiveness of $\gamma$-irradiation in these preparations. $\gamma$-irradiation technique has been previously used to generate nanoscale metals [20] and nanocomposites [21]. Moreover, $\gamma$-irradiation technique has several advantages. Importantly, $\gamma$-irradiation method can produce pure or clean materials without impurities [22]. It is possible to control the size of particles by proper selection of radiation dose etc.

Trial studies have also been done for the second phase of RCM to develop functional electrospun polymer nanofibers (F-ESPNF) through radiation induced processes. Functional nanostructures are currently the subject of increasing interest. Importantly, it is fairly easy to include functional properties to the ESPNF by incorporating additives during the electrospinning process. Various types of additives could be incorporated into the ESPNF. Large surface area, excellent moisture/gas transport, extremely low air permeability and unique porous structures of ESPNF are effectively used to improve current technology and applications. Our experimental findings give hopes that ESPNF could be suitably modified by radiation to develop F-ESPNF.
2. EXPERIMENTAL

2.1. Chemicals
Tetraethyl orthosilicate (TEOS, 98%), pyrrole (98%), aniline, gold (III) chloride, ammonium peroxodisulfate (APS), H2PtCl6·H2O and RuCl3·H2O were purchased from Sigma–Aldrich Co. 25% aqueous solution of ammonia and hydrofluoric acid (HF) was obtained from DC Chemicals Co. Poly(N-vinylpyrrolidone) K-30 (PVP) (Junsei Chemical Co., Japan), FeCl3·6H2O, ethanol (Sam-Chunn Chemical Co., Korea) and sodium carbonate (Duksan Pharmaceutical Co., Korea) were used as received.

2.2. Characterization
Field emission scanning electron microscope (FESEM) (Hitachi, S-4700, Japan) and high-resolution transmission electron microscope (HRTEM) (JEOL, JEM-2010, USA) have been used to find particle size and morphology. EDAX spectrum was recorded simultaneously with the measurement of FESEM. The specific surface area of HSNCs-PPy/PtRu catalyst was determined using a porosimeter (D.A MOUNTECH, Macsorb HM model-1201, Japan).

2.3. Synthesis of HSCNC(PPy/MNP)
Scheme 1 illustrates the steps involved in the preparation of HSNC(PPy). The steps include the preparation of (a) spherical silica (SiO2) particles, (b) coating the surface of the SiO2 particles with a layer of poly(vinylpyrrolidone) (PVP) and adsorption of Fe3+ ions over the layer of PVP, (c) polymerization of pyrrole to obtain SiO2-PPy (core-shell) composite, (d) dissolution of SiO2 (core) to obtain HSNC(PPy) and (e) loading of MNP onto HSNC(PPy) through γ-radiation to obtain HSCNC(PPy/MNP).

2.3.1. Preparation of SiO2 template
Particles of SiO2 (in the size range of 500 nm) were synthesized as detailed elsewhere [23]. Briefly, to a solution containing 1.0 L of ethanol and 80 mL of deionized water, 40 mL of aqueous solution of ammonia (25%) was added. 60 mL of TEOS was added into the mixture under vigorous stirring. The reaction mixture was stirred for 12 h at 25°C and spherical SiO2 particles were obtained.

2.3.2. Preparation of SiO2-PPy (core-shell) composite
Spherical SiO2 particles were dispersed into ethanol (30 mL) and sonicated. PVP (2.5 g) was added into the dispersion and stirred overnight to adsorb PVP on the surface of SiO2 particles. The PVP-protected SiO2 particles were dispersed into an aqueous solution containing FeCl3·6H2O (5.0 g). Then, pyrrole (1.1 mL) was added into the solution containing Fe3+ ions adsorbed PVP-protected SiO2 particles and stirred for 12 h. The black colored SiO2 (core)-PPy (shell) composite was centrifuged, washed several times with distilled water and dried in an oven at 60°C for 8 h.

2.3.3. Preparation of HSNC(PPy)
SiO2 was etched out from SiO2 (core)-PPy (shell)/MNP composite by treatment with HF [24].

2.3.4. Preparation of HSCNCs(PPy/MNP) by γ-radiation
A solution containing 188 ml of water and 12 ml of 2-propanol was prepared. 0.5 g of HSNCs-PPY was dispersed into the water : alcohol solution containing 0.21 g of H2PtCl6·H2O and 0.21 g
of RuCl₃·H₂O. Nitrogen gas was purged into the solution for 30 min and subsequently subjected to γ-radiation (60Co source) at ambient temperature. A total irradiation dose of 30 kGy (dose rate = 6.48×10⁵ h⁻¹) was applied. HSCNs (PPy/MNP) was washed repeatedly with distilled water, centrifuged and dried in an oven at 60°C.

2.4. Synthesis of HSCNC(PANI/MNP)
We have utilized two approaches for the preparation of HSCNC(PANI/MNP) using γ-irradiation.

In the first approach, γ-irradiation was used to load MNPs (Au) on to the PANI shell coated over SiO₂ template. PANI coating (shell) on SiO₂ (template) surface was performed by oxidative polymerization of aniline in the presence of SiO₂ (template). In a typical procedure, PVP coated SiO₂ particles were dispersed in a solution of 0.1 M APS for 1 h. The APS adsorbed SiO₂ particles were filtered and put into a solution containing 50 mM of aniline in 0.5 M HCl and stirred for 2 h. The PANI coated SiO₂ (silica-PANI(shell)) was separated and dried at 60°C. Calculated amount of SiO₂-PANI(shell) particles were dispersed in a water and isopropyl alcohol (15:1 v/v) mixture solution containing 2 x 10⁻³ M HAuCl₄. The mixture was irradiated by γ-ray (60Co source at ambient temperature for a total radiation dose of 30 KG. The MNP(Au) loaded silica-PANI(shell) was filtered, washed with water and dried.

In the second approach, γ-irradiation was used for the formation of both PANI (shell) and MNPs (Au). In a typical experiment, a mixture solution containing aniline (50 mM), HAuCl₄ (1 x 10⁻³M) and silica-PVP (0.2g) particles was irradiated with γ-irradiation. The green mass silica-PANI(shell)/MNP(Au) composite was filtered, washed with water and dried.

3. RESULTS AND DISCUSSION

SEM images in different magnifications (Fig. 1a and b) show that SiO₂ particles (hard template) are spherical with nearly uniform diameters in the range of 400-420 nm [25]. FESEM images of SiO₂-PANI core-shell composite (Fig. 1c and d) particles show the existence of PANI coating with a thickness of 30-40 nm on the surface of SiO₂. Thus, we ensured that PANI layer is present as shell on the surface of SiO₂. FESEM images (Fig. 1d and e) of silica(core)-PANI(shell) loaded with AuNPs are presented. AuNPs of sizes in the range 30-50 nm are found on the surface of PANI(shell). EDAX of silica(core)-PANI(shell)-MNP composite confirms the presence of Au (Fig. 2). Silica (core)-PPy (shell)/MNP composite also showed similar features.

We have authenticated the formation of conducting polymer based HSCNCs by removing the core (silica). Typically, HRTEM image of HSCNC(PPy/MNP) (Fig. 3a and b) reveals that PdPt nanoparticles (MNPs) were distributed on both sides of the HSNC-PPy. For comparison, HRTEM image of the HSCNC(PPy/PtRu) prepared by conventional chemical reduction (Fig. 3b) is presented. In this case, PtRu nanoparticles are present only on the outer surface of the HSNC(PPy).

In the literature, chemical and physical methods have been utilized for the preparation of metal or alloy particles in nanometer sizes. The method include photolytic reduction [26], solvent extraction [27] reduction in aqueous/nonaqueous [28, 29]. A close analysis of literature related to the loading of MNPs onto different matrices reveals the following. The chemicals used for the reduction of metal ions generally leave residual impurities, which generally decreases the activity of MNPs.
Also, stringent post-treatment procedures are needed to remove the impurities from the catalyst system to have good electroactivity of MNPs. The loading of “impurity free” MNP onto polymer support is a technical challenge. It is beneficial to avoid chemicals for the reduction process in the preparation of ‘clean’ MNPs or to prefer a reduction process which will not result products as impurities to the MNPs. Radioysis has been proved as a convenient technique for the preparation of size-controllable MNPs [30] because hydrated electrons generated from water have very strong reduction capability towards metal ions. Gamma radiation (γ) has been effectively used for the synthesis of MNPs because reduction of metal ions could be achieved at ambient conditions [31].

In the present work, γ-radiation induced reduction of metal ions has been specifically selected. The choice of γ-radiation for reduction is based on effectiveness, in producing ‘clean’ catalyst particles, on both sides (inner and outer) of the HSNC(PPy). Further, the Pt–Ru nanoparticles were formed by γ-radiation at room temperature. There are two striking advantages on using γ-radiation for the preparation HSCNCs(PPy/PtRu). First, clean PtRu nanoparticles were loaded onto the HSNCs(PPy) as compared to the chemical and other routes. The reduction of metal precursors by conventional reducing agent result in impurities and often need repeated purification for the removal of the adhered impurities from MNPs. However, in the case of γ-radiation route, impurity generating reducing agent is not required for the preparation of MNPs. Further, smaller sizes of MNPs were obtained by γ-radiation induced reduction. Further, γ-radiation can penetrate into the surface of HSNCs(PPy) and can result in distribution of PtRu nanoparticles on both sides of the hollow spheres. We have employed γ-radiation for obtaining uniform distribution of PtRu nanoparticles into HSCNs(PPy) to obtain HSCNC(PPy/MNP) catalyst.

We also wanted to ensure the importance of γ-irradiation for the preparation of CP based HSCNCs. Towards this, we have employed γ-irradiation for the preparation of conducting polymer shell(PANI) as well for simultaneous loading of MNP (Au). Fig. 4 shows the FESEM image of silica(core)-PANI(shell)/Au composite prepared by the simultaneous formation of PANI(shell) and MNPs using gamma radiation. FESEM images reveal that the thickness of PANI layer is around 60-70 nm. Thus, γ-irradiation induced aniline polymerization could result much higher thickness for PANI shell. It is to be noted that chemical polymerization of aniline resulted a layer thickness of around 50 nm. Also, γ-irradiation induced formation of PANI layer (shell) and MNP (AuNPs) resulted larger number of smaller sized AuNPs (Fig.4).

We have also carried out feasibility studies on the preparation of F-ESPNF. The methodology adopted for the preparation of F-ESPNF by radiation is briefly mentioned (Scheme 2). Typically, new poly(vinylidene fluoride) based multi-functional conductive nanoweb membranes were prepared. Gold nanoparticles(AuNPs) were then loaded into the PVdF-Si NFMs by gamma radiation induced reduction method. Thus, new functional conductive nanowebs, PVdF-@AuNP-NWs were prepared. The interesting features of ESPNFs, such as their flexibility for physical/chemical modification/functionalization, possibility to induct multi-functionalities and unique physical properties, can be effectively utilized for applications in areas that include biotechnology, biosensors, separation science, batteries etc [32-34]. The large surface area to volume ratio and the reusability of electrospun nanofibers/nanowebs provide a basis for generating new multi-functional nanowebs. We anticipate the new F-ESPNF would find application in biosensors and biomedical fields.
REFERENCES

FIG. 1. FESEM images (a and b) SiO$_2$ particles, (c and d) SiO$_2$ (core)-PANI(shell) composites and (e and f) SiO$_2$(core) – PANI(shell)/MNP composite prepared by $\gamma$-irradiation.
FIG. 2. FESEM image (a) and EDAX spectrum (b) of SiO2(core) – PANI(shell)/MNP composite

FIG. 3. EF-TEM images of HSCNCs(PPy/MNP) prepared by (a) γ-irradiation (b) chemical reduction
FIG. 4. FESEM images of SiO2(core) – PANI(shell)/MNP composite prepared by one-step gamma irradiation method.
Scheme 1. Steps involved in the preparation of HSCNCs(PPy/MNP) catalyst.

Scheme 2. Steps involved in the preparation of multifunctional electrospun nanowebs
RADIATION SYNTHESIS OF PEGDA AND ACRYLATED PALM OIL NANOSIZED GEL FOR BIOACTIVES IMMOBILIZATION


Summary
The use of microemulsion in the development of nanosized gel based on polyethylene glycol diacrylate (PEGDA) and acrylated palm oil (APO) is demonstrated. PEGDA was solubilized in n-heptane with use of AOT at 0.15M concentration to form reverse micelles, while APO was solubilized with SDS in water to form direct micelles. Both of these systems were depicted by means of ternary phase diagram. These micelles were than irradiated at 1,3,5,10,15 and 30kGy using gamma irradiation or EB to crosslink the entrapped polymer in the micelles. Ionizing radiation was imparted to the emulsions to generate crosslinking reactions in the micelles formed. The nanosized gel was evaluated in terms of particle diameter using dynamic light scattering and the images of the nanosized gel were studied using transmission electron microscopy (TEM). Results show that the size, charge and shape of the particles are influenced by concentration of surfactants and radiation dose. This study showed that this method can be utilized to produce nanosized gel. Future work include the attachment of functional group to the nano sized gel, loading of drug such as curcumin and further characterization using dynamic light scattering.

Method A: Synthesis Of Polyethylene Glycol Diacrylate Nanogel Using Irradiation Of Inverse Micelles Technique
The applications of nanogel in biomedical fields have been a subject of interest in recent years due to extensive development of bioactive materials. While it is use for targeting specific cell type, it also known to act as a protection layer to the bioactive macromolecules it contained from enzymatic degradation [1]. There are various possible ways to synthesize such system including the classical chemistry route [2-3] or radiation route [4]. The latter have proven to be advantageous in term of safe of use in biomedical applications due to non involvement of hazardous chemical additives. This work is aimed to synthesize nanogel from irradiation of water in oil (W/O) micelles from polyethylene glycol diacrylate (PEGDA) in n heptane. The nanogel will then be functionalized with vinylpyrrolidone (VP) and N-isopropylacrylamide (NIPAAM) and immobilized with curcumin for delivery purposes.

Inverse micelles or also known as reverse micelles are micro emulsion that involves three or more components in the system. The external part of inverse micelles is made up of the more hydrophobic part of a surfactant (hydrocarbon), while the more hydrophilic (polar or charged) part is directed to the internal part of the aggregates. The system can be applied to impregnate hydrophilic polymers, thus is useful for synthesis of nano polymeric gel. The final size of inverse micelles can be adjusted by varying the molar ratio of water to surfactant. The more surfactant is used, the smaller the micelles will be due to suppression of hydrophobic part of the surfactant inward the aggregates.

Preparation of inverse microemulsion
Emulsions were prepared by dissolution of PEGDA in ultrapure water, stirred and filtered with 0.45μm (Minisart, Sartorius). To 100ml of n heptane containing 0.05 to 0.25 of AOT, 10ml of PEGDA was added. The size of emulsion formed was measured by using dynamic light scattering and TEM. In this work, the smallest size of emulsion was used (figure 1a and 1b).
Irradiation of inverse micelles containing PEGDA

The mixture mentioned above was then irradiated at 1, 3, 5, 10, 15 and 30kGy with 3MeV of voltage and 5mA of beam current (figure 2). All the mixtures were saturated with argon gas prior to irradiation. The nanosized gel formed during the irradiation process was then recovered by evaporation of n heptane using rotary evaporator and precipitation of the dry mass with mixture of acetone and methanol (9:1). The precipitate was washed 5 times with the acetone/alcohol mixture to remove excess of AOT. The nano sized gel was than measured by using dynamic light scattering (figure 3).

After irradiation of the microemulsion with electron beam no physical change was observed at 1 and 3kGy. At 5kGy cloudy liquid was formed, while for 10, 15, 20 and 30kGy precipitates were observed in the bottom of the vessels.
FIG. 3. Gel are formed at 5kGy and above, size increased was observed at higher dose.

Figure 4 shows TEM image of the nanosized gel obtained from irradiation of the inversed micelle of PEGDA. This nanosized gel will be functionalized with addition of VP and NIPAAM and loaded with curcumin as model drug. Further characterization with static light scattering will also be done.

Method B: Nanosized Gel from Acrylated Palm Oil Microemulsion Using Radiation Technique
Various nanostructure polymers have been devised in drug delivery research. Over the past few decades and with the advances in nanoscience and nanotechnology, researchers showed interest in developing biodegradable nanoparticles as drug delivery devices. In this case, natural polymers such as vegetable oils (palm oil and soybean oil) are the potential materials and have been used to synthesis nanoparticles. The nanoparticles in the form of micellar solutions consisting of small
particles of 10-400nm diameters, called polymeric micelles showed great promise as potent vehicles for controlled drug delivery [5].

**Preparation of microemulsion**
The micellar system was prepared based on oil in water (O/W) and was created using a ternary phase and solubilization diagrams. The solubilization of these three basic components, i.e., oil (APO), water and surfactant were observed to determine the micro/nanoemulsion regions as illustrated in Figure 5.

![FIG. 5. Ternary Phase Diagram of Water/SDS/APO.](image)

Different concentrations of APO, i.e. approximately at 2% (noted as a) and 0.2% (noted as b), with different concentration of the surfactant (above and below SDS cmc region) in an aqueous solution were prepared. These samples were used for size and stability measurements.

![FIG. 6. Solubilization images of the (Water/SDS/APO) systems a) Emulsion systems and b) Microemulsion systems.](image)

Two main properties of APO in water system, (Water/SDS/APO) have been found. First property is known as emulsion, as shown in Fig.6a. Emulsion can be described as unstable solution and has been visualized as two layer phases. In this system, unstable molecules undergo coalescent forms creaming layer (upper phase) and turbid layer (bottom phase). Meanwhile, another property is more isotropic, homogeneous and semi transparent, as shown in Fig.6b. This system is known as microemulsion. Their appearance can be determined at the solution that has less amount of polymer content. These microemulsion were then used for further analysis.
Particle sizing
The samples were filtered using a disposable PTFE Teflon filter for removing of suspended materials or impurity. The sizes of the micelles were determined by photon cross correlation spectroscopy (PCCS) using a dynamic light scattering (Sympatec Nanophox) at a wavelength of 632 nm.

Radiation synthesis of nanoparticles
Subsequently, the micelles which are in the form of microemulsions were irradiated at different doses using a gamma radiation at 1, 5, 10, 15 and 25 kGy. After irradiation, the irradiated micelles were subjected to sizes determination.

Transmission Electron Microscopy (TEM)
Transmission electron microscopy was performed using a Zeiss microscope (Jeol, Japan), 120 keV, with magnification of 30000X for the measurement of emulsion (before irradiation) and gel sizes (after irradiation). Figure 7 shows the effect of surfactant concentration, in this case, 0.008M SDS is simply not enough to separate the APO while addition 0.01M SDS is adequate for forming of good micelles.

![Image of TEM images](7a) ![Image of TEM images](7b)

*FIG. 7. a) Water/SDS 0.008M/1.8% APO emulsion image. b) Water/SDS 0.01M/1.8% APO emulsion image. Both at 120keV. X30000.*

Figure 8 shows the effect of irradiation at 25kGy of APO emulsion at 0.008M and 0.01M of SDS. The use of surfactant gives better result in formation of nanosized gel where more define gel was obtained with higher amount of surfactant.
Conclusion for Method A and B
PEGDA nanogel can be produced via irradiation of AOT micelles containing the polymer
The size of micelles can be controlled by varying the amount of AOT
The size of gel can be controlled by varying the irradiation dose
APO can be synthesized and developed into micro and nano sized particles using ionizing radiation technique in microemulsion system.
Surfactant Sodium Dodecyl Sulphate (SDS) showed good solubility to APO and (Water/SDS/APO) systems can be used to produce nanoparticles with irradiation.

Future work
Further characterization of the system molecular weight growth and size using static light scattering
Grafting of functional polymer such as VP and NIPAAAM
Incorporation of curcumin as drug for possible drug delivery applications
REFERENCES


PH-SENSITIVE NANOGELS SYNTHESISED BY RADIATION-INDUCED CROSS-LINKING OF HYDROGEN-BONDED INTERPOLYMER COMPLEXES IN AQUEOUS SOLUTION


1. INTRODUCTION

Nanogels, i.e., internally cross-linked hydrophilic polymeric particles of sub-micron sizes, gained much interest over the last years due to their possible application as components of advanced type of medicines, like drug carriers.\(^1\)\(^-\)\(^3\) It is expected that they can facilitate distribution and delivery of different types of biologically active substances (including proteins, peptides and oligonucleotides) in a controlled way within the human body.\(^4\)\(^-\)\(^7\)

Nanogels and their bigger analogues – microgels, are mainly synthesised through free-radical cross-linking polymerization of monomers. This synthetic routine can be carried out in solution but more often emulsion techniques are preferred (mini- or microemulsion) due to easier size control and exclusion of the macrogelation process.\(^8\)\(^-\)\(^10\) Additionally, surfactant-free emulsion polymerization (SFEP) is the method of choice for the preparation of temperature-sensitive particles, mainly based on poly(N-isopropylacrylamide).\(^11\)\(^,\)\(^12\) Nanogels were also successfully prepared by intramolecular cross-linking of single macromolecules.\(^13\) More recently, covalent stabilization was utilized to obtain the self-assembled structures like micelles of amphiphilic block copolymers, held by relatively weak physical interactions.\(^14\)\(^-\)\(^16\) Due to low stability of these polymeric systems against dilution or temperature changes, different chemistry-based strategies to turn them into permanent nanoparticles were proposed in the literature (e.g., independent stabilization of a core or a shell of the micelles).\(^17\)\(^-\)\(^21\)

A certain disadvantage of the approaches described above is the use of monomers, surfactants or cross-linkers which are usually toxic and have to be removed from the final product by laborious and time-consuming processes. This issue can be important especially if the product is intended for biomedical use. Very often chemical modification of a polymer structure in order to promote cross-linking is also required, what makes the nanogel synthesis a complex, multi-step procedure. Thus, synthesis by non-classical methods where the presence of the mentioned substances can be avoided and eventually the procedure can be reduced to one step seems to be a promising alternative.\(^22\) For example, in our recent series of publications it has been shown that single polymer chains of hydrophilic polymers can be cross-linked in additive-free aqueous solution to permanent nanogels by short pulses of electrons.\(^23\)\(^-\)\(^25\)

Another group of supramolecular polymeric systems are hydrogen-bonded interpolymer complexes (IPCs) which represent a class of pH-sensitive materials with broad potential applications in the field of pharmaceuticals.\(^26\) Extensive studies on the biomedical applications of IPCs were reported by Peppas and co-workers.\(^27\) They have shown that mucoadhesive hydrogels based on methacrylic acid (MAA) and poly(ethylene glycol)’s methacylates can serve as the matrices for controlled release of insulin.\(^28\)\(^-\)\(^30\) The formation of hydrogen bonds between complementary segments in acidic conditions resulted in strong de-swelling of a gel, providing protecting environment for the encapsulated protein against destruction by digestive enzymes. On the other hand, at neutral conditions prevailed in the intestine, the gel particles swelled allowing for controlled release of insulin. PMAA-\textit{graft}\textendash PEG network was also prepared in the form of nanoparticles by precipitation
polymerization and it was suggested that microscopic sizes could provide better up-take through intestine epithelial cells.\textsuperscript{31-33} Synthesis of nanogels comprised of polymers with hydrogen-bonding ability was also elaborated by the Ming Jiang group.\textsuperscript{34} They stabilized micellar aggregates of hydroxyethylcellulose-graft-poly(acrylic acid) by chemical cross-linking, resulting in hollow spheres of 160 - 400 nm size. Sukhishvili and co-workers reported on the synthesis of pH-sensitive, permanently cross-linked micron-sized capsules built through alternate deposition of poly(methacrylic acid) and poly(N-vinylpyrrolidone) on silica or polystyrene particles (so-called layer-by-layer technique) and subsequent removal of the template.\textsuperscript{35} It is also to be noted that nanogels containing hydrogen-bonding components can be prepared by template polymerization of acrylic acid on different matrix polymers and simultaneous/subsequent cross-linking. For example, Lu et al. and more recently Chen et al. obtained nanogels composed of hydroxypropylcellulose and poly(acrylic acid).\textsuperscript{36,37} Moreover, studies on the synthesis of poly(acrylic acid)-gelatin and poly(acrylic acid)-poly(vinyl alcohol) nanogels were also published.\textsuperscript{38,39} In these cases, particles of the semi-interpenetrating type or with the core-shell structure were obtained. It was shown that the stage of hydrogen-bonding between a matrix polymer and a monomer played a crucial role in the successful formation of the nano- or microgels using template method.\textsuperscript{40}

\textbf{Synthesis of PVP–PAA nanogels by radiation-induced cross-linking of their hydrogen-bonded complexes in dilute aqueous solution.}

Irradiation of dilute or moderately concentrated aqueous solutions of polymers leads in the first order to the radiolysis of water molecules, in accordance with a principle that absorption of radiation energy is directly proportional to the weight fraction of the components. The primary products, namely hydroxyl radicals, hydrogen atoms and solvated electrons, can react further with the macromolecules present in solution.\textsuperscript{41} One of the most reactive species towards the simple saturated polymers are hydroxyl radicals (\textsuperscript{•}OH) which react with the macromolecules through abstraction of hydrogen atoms from their chains. In the absence of oxygen the formed polymer radicals can undergo one- (degradation or hydrogen transfer) or two-radical (disproportionation or cross-linking) processes. From the point of view of the network formation, the most important reaction is, of course, cross-linking which can proceed in either inter- or intramolecular manner. It was shown that by choosing appropriate irradiation conditions and concentration of a polymer in solution one can regulate the efficiency of both processes. If the concentration of a polymer is higher than a critical overlapping limit and the average number of radicals formed on a chain is lower than 1, intermolecular recombination occurs preferably and can finally lead to the formation of macroscopic gel (if cross-linking dominates over chain scission).\textsuperscript{41} On the other hand, in dilute solutions irradiated at high dose rates (thus, many radicals are present simultaneously on a single chain), intramolecular reaction dominates over the intermolecular one. Recombination of radicals within a single macromolecule leads to the formation of a new, internally cross-linked structure with similar size but different properties in comparison to the free polymer chain. To distinguish this new macromolecular type of architecture it was called a nanogel.\textsuperscript{24} All processes described above are schematically presented in Figure 1.
Before irradiation, PVP–PAA aqueous solutions were saturated with N$_2$O. This manipulation doubles the radiation-chemical yield of hydroxyl radicals $G = 2.8 \times 10^{-7}$ mol J$^{-1}$ due to the following reaction:

$$\text{e}_{aq}^- + \text{N}_2\text{O} \rightarrow \text{H}^+ + \cdot \text{OH}$$  \hspace{1cm} (1)

For the preparation of hydrogen-bonded interpolymer complexes poly(\textit{N}-vinylpyrroldione) (Kollidon, BASF) of weight-average molecular weight $M_w = 650$ kDa and two poly(acrylic acid)s of $M_w = 5$ kDa (Polyscience Inc.) and 50 kDa (Aldrich), denoted further as PAA5 and PAA50, were used. Detailed characteristics of these polymers are given in Table 1. All other chemicals were of analytical quality and were used as received.
TABLE 1. CHARACTERISTICS OF THE POLYMERS USED IN THE STUDIES: MN AND MW FOR PAA5 ARE NOMINAL VALUES, MN AND MW FOR PVP WERE DETERMINED IN WATER USING GPC EQUIPPED WITH LIGHT SCATTERING DETECTOR, VALUES OF RG AND RH WERE DETERMINED BY MULTIANGLE LASER LIGHT SCATTERING, APPARENT PKA VALUES WERE OBTAINED BY POTENTIOMETRIC TITRATION; N. D. MEANS NOT DETERMINED.

<table>
<thead>
<tr>
<th>Parametr sample</th>
<th>$M_n$ / Da</th>
<th>$M_w$ / Da</th>
<th>$M_w / M_n$</th>
<th>$R_g$ / nm</th>
<th>$R_h$ / nm</th>
<th>apparent $pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAA5 (Polyscience Inc.)</td>
<td>$2.1 \times 10^3$</td>
<td>$5.0 \times 10^4$</td>
<td>2.4</td>
<td>n. d.</td>
<td>n. d.</td>
<td>6.37</td>
</tr>
<tr>
<td>PAA50 (Aldrich)</td>
<td>n. d.</td>
<td>$4.9 \times 10^4$</td>
<td>n. d.</td>
<td>19.1</td>
<td>13.0</td>
<td>6.68</td>
</tr>
<tr>
<td>PVP (Basf)</td>
<td>$2.2 \times 10^5$</td>
<td>$8.0 \times 10^5$</td>
<td>3.6</td>
<td>49.5</td>
<td>29.5</td>
<td>---</td>
</tr>
</tbody>
</table>

Prepared IPC solutions, according to procedure described by Henke et al\textsuperscript{42}, were irradiated at pH = 2.8, 3.0 or 3.4 depending on the polyacid used and aggregation characteristics of the complexes. The N\textsubscript{2}O-saturated PVP–PAA solutions in glass reactor were placed in front of the linear electron accelerator and irradiated with different doses, namely 2, 5, 8, 10, 12 and 15 kGy. This corresponded to the total amount of generated hydroxyl radicals in solution from ca. 1 mM for the lowest dose to ca. 8.5 mM for the highest one. Covalent stabilization of PVP–PAA complexes was followed by analysis of the irradiation products by laser light scattering at pH 10, as at these conditions no stable hydrogen bonds are formed between both polymers (e.g., IR result in Figure 2). Thus, if intra-complex cross-linking did not take place in the system, the molecular weight and the size should remain at the level typical for non-irradiated samples (dose = 0). In order to limit the influence of aggregation process, irradiation experiments were performed 24 hours after preparation of PVP–PAA solutions. Changes of $M_w$ as a function of absorbed dose for PVP, PAA50 and PVP–PAA solutions are presented in Figure 2.
FIG. 2. Changes of the apparent weight-average molecular weight $M_w$ at pH = 10 as a function of total absorbed dose by $N_2O$-saturated PVP, PAA50 and PVP–PAA solutions. pH values in the legend correspond to the irradiation conditions.

Separate irradiation of PVP and PAA aqueous solutions at the chosen concentration conditions should mainly lead to the intramolecular cross-linking of their chains. However, the increase of PVP molar mass indicates also a presence of the intermolecular recombination. As we reported in our previous studies, the latter process cannot be totally eliminated and it always competes with intra-type process. In the nanogel synthesis from simple hydrophilic polymers, the contribution of the former reaction to the total chemical-radiation yield of recombination is usually not higher than 1-2 %. In PAA50 solutions the molecular weight remained unchanged because poly(acrylic acid) at pH = 3.0 is not fully protonated and in consequence negative charges contributes to the electrostatic stabilization of its chains, retarding cross-linking reactions.

As seen for $D = 0$, aggregates of PVP–PAA interpolymer complexes were unstable at pH = 10 and disintegrated to individual components, giving the values of molar masses in the range of 600-800 kDa (single PVP chains). Thus, a considerable increase of $M_w$ in PVP–PAA solutions up to 7.5 kGy was ascribed to the intermolecular cross-linking between PVP and PAA chains within IPC aggregates and led to the formation of permanent particles with molar masses between 5 MDa (PVP–PAA5) and 15 MDa (PVP–PAA50). Starting from 10 kGy, $M_w$ did not change significantly what indicates that covalent stabilization of IPC particles reached a certain maximum level.

More information concerning stabilization of PVP–PAA aggregates was provided by the analysis of $R_g$ and $R_h$ changes, both at irradiation conditions (low pH) and at basic pH.
FIG. 3. Changes of the radius of gyration $R_g$ and the hydrodynamic radius $R_h$ as a function of total absorbed dose, measured at (A) $pH = 2.8$ and $3.4$ in $N_2$O-saturated PVP–PAA50 and PVP–PAA5 solutions, respectively, and (B) measured at $pH = 10$.

As presented in Figure 3 A, $R_g$ and $R_h$ measured in PVP–PAA50 solutions at $pH = 2.8$ remained almost unchanged, regardless the irradiation dose. This effect resulted from compact structure of formed particles what limited their further contraction upon intra-complex cross-linking. On the other hand, for PVP–PAA5 system at $pH = 3.4$, $R_g$ and $R_h$ underwent pronounced changes. At the initial stage of the irradiation, i.e., up to $5 \text{kGy}$, $R_h$ and $R_g$ decreased almost by half and reached values typical for single interpolymer complex particle. This can be due to two effects. PVP–PAA5 aggregates may have disintegrated partially, probably due to vigorous solution mixing in the preparative pulse radiolysis experiments. One can also assume that observed size decrease could be induced by pronounced intra-complex cross-linking. For instance, we observed a two-fold decrease of PAA size upon irradiation of their macromolecules in our earlier studies.\textsuperscript{24} However, as compact particles contain less water in their interior (around 50 -60 % when compared to 90-95 % in case of the polymeric coil),\textsuperscript{43} it is difficult to predict such a strong contraction of a complex structure upon irradiation. Further irradiation of PVP–PAA5 solutions caused an increase of the radius of gyration and $R_g/R_h$ ratio close to a uniform sphere model (the latter parameter changed from $\sim 0.58$ at $0 \text{kGy}$ to $\sim 0.69$ at $15 \text{kGy}$), indicating that cross-linking was taking place in the whole particle volume.
PVP–PAA aggregates were further analysed in the swollen state. In Figure 4 B, changes of the hydrodynamic radius and the radius of gyration at pH =10 are presented for PVP–PAA5 and PVP–PAA50 systems irradiated at the same conditions as above. The initial increase of the hydrodynamic radius was induced by particle swelling what emphasizes a fact of covalent stabilization of IPCs. For the aggregates containing shorter polyacid one can clearly observe a decrease of $R_h$ and $R_g$ as a function of absorbed dose, pointing an increasing cross-linking density within the particle. On the other hand for IPCs composed of PVP and PAA50 chains cross-linking process was more complex. While the radius of gyration increased, the hydrodynamic size of irradiated particles first increased and then levelled off. This fact indicates that cross-linking took part in the shell and in the core of the particle.

**FIG. 4.** $R_g/R_h$ ratio at pH =10 as a function of total absorbed dose for N$_2$O-saturated equimolar PVP–PAA solutions. PVP–PAA5 and PVP–PAA50 solutions were irradiated at pH = 3.4 and 2.8, respectively. Total concentration of the polymers in solution was equal to 0.01 mol dm$^{-3}$. 

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The difference between cross-linked and uncross-linked particle is further seen on the basis of structural parameter changes (Figure 4). For non-irradiated samples, values of □-parameter are between 1.6-1.8, which is slightly lower than a typical range for a polydisperse polymer coil in a good solvent, however reflected dissociation of a complex. With increasing dose, one can clearly observe a drop of the $R_g/R_h$ ratio which in the later stage of irradiation process reaches a value typical for uniform sphere in the case of PVP–PAA5 system and core-shell for PVP–PAA50. Thus, the initial structure of PVP–PAA50 complexes was “frozen” upon irradiation. On the other hand, for complexes containing shorter PAA chains, the value of the structure parameter is a consequence of dissociation of the clusters into single complexes. This effect is not apparent and was observed every time when irradiation was performed.

FIG. 5. (A) Tapping-mode phase AFM picture of PVP–PAA5 nanogels cast from aqueous solution of pH = 10 (irradiated at pH = 3.4 with 15 kGy) on mica surface and (B) intensity-averaged distribution of hydrodynamic diameter obtained by dynamic light scattering at 90°. Total concentration of the nanogels was equal to 0.1 g dm⁻³.

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PVP–PAA5 nanogels from solution of pH = 10 were additionally visualized by Atomic Force Microscopy. In Figure 5 spherical particles with sizes between 60 and 90 nm can be observed as well as a fraction of smaller particles with diameters around 20-30 nm. In the distribution of the hydrodynamic diameters one can observe a maximum around 100 nm. Taking into account that distribution obtained from dynamic light scattering is intensity-averaged, particles with bigger size contributed more than the smaller ones. Secondly, particles cast on mica surface can be partially dehydrated and thus smaller sizes in AFM than in light scattering measurements were to be expected.

ACKNOWLEDGEMENTS

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REFERENCES

RADIOLITICALLY SYNTHESIZED HYBRID NANOSYSTEMS FOR BIO-NANO-TECHNOLOGIES

A. Krkljes, SERBIA

Summary

In this report a review of the main results and the studies carried out under the scope of the IAEA CRP project: Nanoscale Radiation Engineering of Advanced Materials for Potential Biomedical Application is presented. In particular two topics are discussed: radiation synthesizing of Ag nanoparticles in hydrogels for potential biomedical application and decoration of carbon nanotubes with Ag clusters by gamma irradiation.

1. CURRENT STATUS OF THE WORK ON NANOTECHNOLOGY WITH APPLICATION OF IONIZING IRRADIATION FOR SYNTHESIZING OF Ag NANOPARTICLES IN HYDROGELS FOR POTENTIAL BIOMEDICAL APPLICATION

Research personnel:
Dr Zorica Kacarevic-Popovic Vinca Institute of Nuclear Sciences, Dr Aleksandra Krkljes Vinca Institute of Nuclear Sciences, Jelena Krstic PhD student Vinca Institute of Nuclear Sciences, Jelena Spasojevic PhD student Vinca Institute of Nuclear Sciences, Zeljka Jovanovic PhD student TMF Belgrade, Prof dr Vesna Miskovic-Stankovic TMF Belgrade University, Doc dr Simonida Tomic TMF Belgrade University, Prof. Dr Srdjan Popovic, Faculty of Medicine, Belgrade University, Prof Dr Miodrag Colic, Faculty of Medicine Belgrade University, Ass Dr Mirjana Dragaševic, Faculty of Medicine Belgrade University

1.1. The aim of the work

The aim of the work is systematically developing synthetic strategies for incorporation of nano-Ag in hydrogel networks by gamma irradiation, using liquid filled cavities in hydrogels as nanoreactors (template synthesis), and exploring favourable characteristics of radiation technology for nanoscale engineering of materials especially for biomedical application such as easy process control and the possibility of joining synthesis and sterilization in one technological step. The radiation technique does not require any extra substances, and does not need any further purification. The incorporation of template technologies with the synthesis techniques such as gamma ray irradiation methods is expected to produce more elaborated Ag nanoparticles and more diversified nanoelevated Ag suitable for biomedical applications. Although gamma irradiation has proven to be a powerful tool for synthesis and modification of materials, not so many studies have been reported concerning the radiolytic formation of metal nanoparticles in hydrogel matrix.

The chosen hydrogels, being previously synthesized or crosslinked by gamma irradiation, are suitable for various applications in reconstructive surgery, including wound dressing, tissue expanders etc. Nano-Ag is successfully incorporated in hydrogel matrix such as PVA, and poly(BIS-co-HEMA-co-IA) copolymer hydrogel, as has been reported by KACAREVIC-POPOVIC Z. and co-workers (2007)\(^1\) and KRKLJES A. and co-workers (2007)\(^2\). It was shown that PVA* radicals have sufficient reducing ability to produce Ag nanoparticles in swollen polymer matrix. Further work on antibacterial properties of these materials is in progress. In addition, developing of thermal responsive and pH responsive hydrogel nanocomposites is in progress.
1.2. Synthesis of nanocomposites

The processes of hydrogel formation are essentially due to monomer polymerization or crosslinking of preformed polymers. A great variety of methods to promote crosslinking have indeed been used to prepare hydrogels including chemical and physical methods. Among these, high-energy radiation, in particular gamma rays and electron beam, can be used to polymerize unsaturated compounds. This means that water-soluble polymers can be converted into hydrogels using high-energy radiation. Rosiak and coworkers have presented a successful methodology of hydrogel dressing production, FECHINE and co-workers (2004), based on high-energy radiation. These hydrogels are prepared through irradiation of poly(N-vinyl-2-pyrrolidone) (PVP) aqueous solutions. Hydroxyl radicals formed during water radiolysis react with PVP generating macroradicals. One of the resulting reactions from these radicals is recombination leading to crosslinks.

![Reactants used in the synthesis of the investigated systems.](image)

Reactants used in the synthesis of the investigated systems in this work, and their general chemical structure are shown in Fig. 1.

The gels were polymerized or crosslinked by gamma irradiation radical copolymerization or crosslinking. The reaction mixture was degassed prior to polymerization and placed between two glass plates, sealed with a spacer. The solutions were irradiated in a $^{60}$Co radiation source, under ambient conditions, to absorbed dose of 25 kGy. In order to remove any uncrosslinked polymer molecules, the hydrogels were immersed in deionized water, which was changed every day, for one week.

Ag/hydrogel nanocomposites were prepared by swelling the crosslinked polymer samples with water solutions of AgNO$_3$ and 2-propanol. Swelling of Ar-saturated gels was carried out in tightly
closed containers for 48 h at room temperature in the dark (longer swelling period had no effect). In the second step, Ag⁺ ions were reduced in hydrogel using electron transfer reactions from radical species formed in water radiolysis. Gamma irradiation was performed in 60Co radiation facility, at room temperature, until achieving complete reduction of Ag⁺ ions.

1.3. Characterization of nanocomposites
Absorption spectra of Ag/hydrogel nanocomposites were recorded in the wavelength range from 300 to 800 nm using Thermo Scientific Evolution 600 spectrophotometer.

Transmission electron microscopy (TEM) measurements were performed using a JEOL 100CX instrument, which has an operating voltage of 100 kV.

The X-ray diffraction (XRD) measurements of the Ag/hydrogel nanocomposites were performed on Bruker D8 Advance Diffractometer (Cu Kα1 radiation, λ = 0.1541 nm).

For the swelling studies, the xerogel discs were immersed in an excess of Kokubo's Simulated Body Fluid (SBF) solution, KOKUBO and co-workers (1990)⁴, with the pH value of 7.1, to obtain equilibrium swelling at 37 °C. SBF is an acellular simulated body fluid that has inorganic ion concentrations similar to those of human extracellular fluid.

The release of Ag⁺ ions from Ag/hydrogel nanocomposites was determined by atomic absorption spectroscopy, by Philips-Pyu Unicam SP 9 AAS spectrophotometer.

1.4. Results and discussion
The primary products of water radiolysis are shown in Eq. (1):

\[
\text{H}_2\text{O} \rightarrow e_{\text{aq}}^- (2.7), \text{OH}^* (2.7), \text{H}^+ (0.6), \text{H}_2 (0.45), \text{H}_2\text{O}_2 (0.7).
\]  

(1)

The numbers in parentheses represents the respective G values. The G value for a given irradiated system is the absolute chemical yield expressed as the number of individual chemical events occurring per 100 eV of absorbed energy. Thus the G (e_{aq}^-), G (OH^*), etc. are the number of solvated electrons, hydroxyl radicals, etc., formed per 100 eV of absorbed energy. In the presence of alcohol, the OH^* and H^* radicals abstract hydrogen from the alcohol to produce an alcohol radical. It is well known that the radiation crosslinking of polymer (POLYM) molecules is mainly induced by OH^* radicals in aqueous medium (with the G value of irradiation-induced intermolecular crosslinking 0.48):

\[
2 \text{POLYM (H)} + 2\text{OH}^* \rightarrow \text{POLYM–POLYM (crosslinked polymer)} + 2\text{H}_2\text{O}.
\]  

(2)

Under the given experimental conditions the Ag⁺ ions are reduced with strongly reducing hydrated electrons, 2-propanol radical and the polymeric radicals, formed by H atom abstraction from polymer chains by hydroxyl radicals:

\[
n\text{Ag}^+ + n e_{\text{aq}}^- / (\text{CH}_3)\text{C}^\circ \text{OH}/ \text{POLYM}^* \rightarrow (\text{Ag})_n.
\]  

(3)
After the gamma irradiation, the yellow colored Ag/polymer hydrogel nanocomposites were obtained. Yellow color is characteristic of silver nanoparticles, as shown for Ag/PVP hydrogel nanocomposite on Fig. 2.

![Photograph of PVP hydrogel (left) and Ag/PVP hydrogel nanocomposite (right).](image)

Reduction of Ag⁺ ions in different types of investigated hydrogel matrix yielded the typical surface plasmon of Ag nanoparticles for all investigated systems, with no broad absorptions at wavelengths longer than the particle plasmon band, as shown in Fig. 3. Irradiation of AgNO₃-loaded hydrogels resulted in a strong sharp absorption band centered around 400–430 nm. No significant change in the UV–Vis characteristics of the Ag nanoparticles formed in these hydrogels with compositions was observed. The sharp absorption pattern indicates that the particle size distribution is quite narrow.

The absorption pattern of the xerogel of the same samples, obtained by the evaporation of the water under vacuum, showed the sharp absorption centered at the similar wavelength range. In the dry polymer matrix (xerogel) of all polymer compositions, the signal not only increased in intensity compared to the hydrogel, but also shifted as a consequence of change in dielectric properties of surrounding environment (Fig. 3, example for Ag/PVA hydrogel and xerogel nanocomposites).

![The appearance of the surface plasmon band in the investigated nanocomposite systems.](image)
To probe into the morphologies and size of Ag nanoparticles, TEM micrograph of Ag nanoparticles are shown in Fig. 4. It is obvious that these Ag nanoparticles like in all investigated systems assume spherical-like morphologies in appearance at nanoscale levels.

![TEM images of AgNPs formed in PVP hydrogel.](image)

**FIG. 4. TEM images of AgNPs formed in PVP hydrogel.**

The XRD patterns of PVP and Ag/PVP nanocomposite are presented in Fig. 5. The XRD pattern for Ag/PVP nanocomposite, as well as in all investigated nanocomposite systems, exactly matched the 111, 200, 220, 331 and 222 Bragg’s reflections from crystal planes of face centered cubic (fcc) structure of silver.

![XRD patterns of PVP and Ag/PVP nanocomposite.](image)

**FIG. 5. XRD patterns of PVP and Ag/PVP nanocomposite.**

The Sherrer diffraction formula was used to estimate the crystalline domain size (d):

\[ d = \frac{k \lambda}{\beta \cos \theta} \]  

(4)

where \( k = 0.9 \) is constant for the cubic structure, \( \lambda = 0.1541 \) nm is the X-ray wavelength, \( \beta \) is the peak angular width and \( \theta \) is the diffraction angle. The crystalline domain size for all systems was found to be about 5-10 nm.

Swelling of the crosslinked polymers in chosen solvent is the most important parameter for swelling studies. Moreover, preliminary studies in buffered solution of pH similar to the pH of biological fluids are very important for the application of hydrogels as biomaterials. In general, a fundamental relationship exists between the swelling of a crosslinked polymer and solvent. The
intake of xerogels, and Ag/xerogel nanocomposite, were followed for a long period of time in SBF solution at 37 °C. Example of plotted swelling curves is presented in Fig. 6.

As can be seen from Fig. 6, swelling capabilities of PVP hydrogels (xerogel) and Ag/PVP hydrogel (xerogel) nanocomposite are increased by time, reaching constant swelling (equilibrium swelling) after a certain period of time. Another observation is that the swelling of the gels is slightly greater for the gel containing Ag nanoparticles, like in all investigated systems.

Sustained, steady supply of active silver is important property of dressing material. From Fig 7. and Fig 8. it can be seen that the release of silver ions (Ag⁺) from nanocomposite systems is continuous during long period of time which means that investigated hydrogel nanosystems meet that criteria.
FIG. 8. Release of silver from Ag/PNIPA hydrogel nanocomposites.

The *in vitro* study of pure hydrogels biocompatibility showed neither evidence of cell toxicity nor any considerable hemolytic activity. Moreover, incorporation of itaconic acid (IA) even increased cytocompatibility of pure hydrogels. Furthermore, the microbe penetration test showed that neither *Staphylococcus aureus* nor *Escherichia coli* passed through the pure hydrogel dressing.

### 1.5. Conclusion

Obtained results indicated that gamma irradiation is suitable for *in situ* generation of Ag nanoparticles in investigated hydrogel matrix by radiolytic products of water. X-ray diffraction analysis confirms the face centered crystal structure of Ag nanoparticles as well as their nanometric size. Swelling properties of synthesized hydrogels, pure and Ag/hydrogel nanocomposites, investigated in the SBF (simulated body fluid) solution at 37 °C exhibits that Ag/hydrogel nanocomposite systems have higher equilibrium swelling compared with pure hydrogel. The release of silver ions (Ag⁺) from nanocomposite systems is continuous during long period of time which means that investigated hydrogel nanosystems meet criteria of sustained, steady supply of active silver.

**THESE RESULTS ARE PUBLISHED IN:**


WORK PLAN

• Second year
  Investigation of antibacterial properties of radiolytically synthesized Ag/hydrogel nanocomposites accordingly to standard protocols.

• Third year
  Synthesis of Au nanoparticles in previously obtained Ag-hydrogel nanocomposites by gamma irradiation. Investigation of biological potential of synthesized AuAg-hydrogel nanocomposites.

• Collaboration with the participants of CRP.

REFERENCES


2. FUNCTIONALIZATION OF CARBON NANOTUBES WITH SILVER CLUSTERS BY GAMMA IRRADIATION

J. Cveticanin, A. Krkljes, Z. Kacarevic-Popovic, O. Neskovic

Since their discovery in 1991 by Iijima, carbon nanotubes (CNTs) have attracted great interest in most fields of science and engineering due to their unique physical and chemical properties. These properties allow them to be applied for a wide range of applications. The major areas of CNTs research are the polymer composites and biomedical materials and devices including biosensors, drug and vaccine delivery vehicles. For the purpose of application of CNTs and also regarding their genotoxicity, functionalization of CNTs takes an important place.

Considerable research efforts have been devoted to the fabrication of Ag nanoparticles supported on CNTs. For the development of new functional materials, γ-irradiation was proven to be a powerful tool. As have been previously shown, the Ag nanoparticles can be easily prepared by the
reduction of metal ions with irradiation. Generally, irradiation induced reactions exhibit the following advantages: the products are free of residual initiators or catalysts and reactions can be conducted at low temperatures and in gaseous, liquid or even solid-state phases. Moreover, γ-irradiation grafting is an efficient and easy method for producing aninorganic–organic materials interface and modifying the surface to form functional composites. The usage of irradiation has been proven as an efficient method to functionlize CNTs with Ag. This study demonstrates the functionalization of CNTs with Ag nanoparticles, achieved via anchoring of the polymer to the surface of CNTs and simultaneous reduction of Ag$^+$ ions under the irradiation. The polymer used in this work was the commercially available polymer, poly(vinylalcohol) (PVA). This paper reports a simple and efficient one-step method of producing a hybrid of uniformly dispersed Ag nanoparticles supported on CNTs without acid purification or using any surfactant or polyelectrolyte to functionalize the tubes. The aim of this work was to investigate the potential of two different chemical routes and reduction ability of different reduction species produced under the irradiation. In general, this work is a part of systematic investigation of potential of radiation technology for nanoengineering of materials, because for nanoscience to become true nanotechnology, there is a need for development in the engineering science of processing at the nanoscale.

**FIG. 1.** The schematic overview of the formation mechanism of Ag/PVA/CNTs under γ-irradiation.
FIG. 2. TEM images of as-prepared Ag/PVA/MWCNTs synthesized by $e_{aq}^-$ and PVA$^\cdot$ (a); PVA$^\cdot$ radicals only (b).
FIG. 3. (a) STM image (76nm×76 nm) of SWCNTs, (b) STM image (47nm×47 nm) of Ag/PVA/SWCNTs (synthesized by $e_{aq}$ and PVA$^\cdot$), (c) STM image (69nm×69 nm) of Ag/PVA/SWCNTs (synthesized by PVA$^\cdot$ radicals only).

**THESE RESULTS ARE PUBLISHED IN:**

MODIFIED CHITOSAN NANOPARTICLE BY RADIATION SYNTHESIS: AN APPROACH TO DRUG DELIVERY AND BIO-BASED ADDITIVE FOR BIOMEDICAL APPLICATIONS

W. Pasanphan, P. Rimduisit, T. Rattanawongwiboon, S. Choofong; THAILAND

Summary
Self-assembly chitosan nanoparticle (CsNP) has been synthesized via radiolytic methodology using gamma irradiation. The systematic condition in preparation was studied. Chitosan nanoparticle was modified using hydrophobic core of deoxycholic acid (DC) and stearyl methacrylate (SMA) and the hydrophilic shell of polyethylene glycol monomethacrylate (PEG). The hydrophobic/hydrophilic CsNP was prepared for drug carrier molecule. The SMA-CsNP was also conjugated with pyperidine, hindered amine light stabilizer function, to achieve a bio-based additive for biomedical plastic.

1. INTRODUCTION

Naturally occurring polymers, especially polysaccharide such as chitosan and alginate, have been in recent years extensively researched as a primary material in forming carriers. Chitosan is a biodegradable polysaccharide derived by partial deacetylation of chitin, which is a copolymer of glucosamine and \(N\)-acetyl-d-glucosamine linked together by \(\beta\) glycosidic bonds [1]. Chitosan can be degraded into \(N\)-acyl glucosamine by general lysozymes in the body, which is subsequently excreted as carbon dioxide via the glycoprotein synthetic pathway [2]. Chitosan has been widely used in pharmaceutical and medical areas, due to its favorable biological properties such as biodegradability, biocompatibility, low toxicity, hemostatic, bacteriostatic, fungistatic, anticancerogen, and anticholesteremic properties [3].

It is well known that polymer micelles have unique core–shell architecture that composed of hydrophobic segments as internal core and hydrophilic segments as surrounding corona in aqueous medium. The hydrophobic core provides a loading space for water-insoluble drugs, whereas the modification of hydrophilic shell affects pharmacokinetic behavior [4]. Additionally, the nano-scaled polymer micelles exhibit many advantages for the use of drug delivery carriers, such as prolonged circulation; tumor localization by enhanced permeability and retention (EPR) effect [5]; and the controlled drug release by using stimuli-sensitive copolymers [6].

Self-assembly through chemical modification is one of the most widely used processes for chitosan nanoparticle fabrication. This aggregation process, caused by incorporation [7] of hydrophobic organic substances into micelles, can play very important roles in adsorption, transfer, and slow release of drug when treating humans. Various hydrophobilized polymers have been synthesized by modifying an organic group on a hydrophilic polymer [8]. Phthaloylchitosan mPEG as a hydrophobic/hydrophilic microsphere with the average size of 1500 nm was developed [9]. Deoxycholate-modified chitosan has widely been proposed as solid capsules for lipid drug carriers. Pang et al. [10] reported their deoxycholate-chitosan with the size of 200–600 nm and it had the ability to encapsulate tocopherol acetate and stearic methyl ester. Kim et al. [11] carried out a study to control the particle size and size distribution of self-aggregates of deoxycholic acid-modified chitosan as a gene delivery carrier within the range 130–300 nm.
Based on the previous reports, chemical modification is the most widely used protocol in preparing hydrophobic/hydrophilic self-assembly chitosan nanoparticles. In this way, the conjugating agent as well as the purification steps is required. In addition, the nanoscale material has been defined to be less than 100 nm [12]. Therefore, the systematic protocol in reducing and controlling the particle size of chitosan still requires further studies to achieve not only the simple and effective preparation protocol but also a superior potential product.

In the present work, radiolytic methodologies to modify chitosan nanoparticle were studied. The modified chitosan nanoparticles are mainly proposed into two medical application purposes, i.e. (i) drug carrier for drug controlled release and (ii) bio-based additive for biomedical plastic. Based on this view point, the systematic examination of fabrication condition of self-assembly chitosan nanoparticle using radiolytic methodology is investigated. Gamma radiation from Co-60 was used as the high energetic radiation in order to produced radiolysis products and subsequently induces radical reaction. This mild process would be expected as a novel simple and effective protocol in preparing self-assembly chitosan nanoparticle containing hydrophobic core with hydrophilic shell as a drug carrier device. The procedure would also be extended to prepare the antioxidant conjugated hydrophobic-modified chitosan nanoparticle as an antioxidant nanofiler for medical plastic, e.g. polylactic acid (PLA).

2. MODIFIED CHITOSAN NANOPARTICLE BASED SELF-ASSEMBLY AS A DRUG CARRIER FOR DRUG CONTROLLED RELEASE SYSTEM

2.1. Systematic preparation study on chitosan nanoparticle formulation via gamma irradiation [13]

In this section, the direct effect of gamma radiation on the particle formulation due to the physical of chitosan was studied. Three route of chitosan formations, i.e.Cs-flake, Cs-colloid, and Cs-acid were used. Fig. 1 indicates the effect of irradiation condition due to chitosan formulation. The observation from TEM gave us information on the particle shape and the determination of particle size. The irradiations of the Cs-flake (Fig. 1(a)) and Cs-colloid (Fig. 1(b)) gave the individual spherical shape with the average size of 95 and 2 nm, respectively. On the other hand, it was difficult to identify the irradiated Chi-acid as a spherical shape. Chitosan particles from Chi-acid were found in variable shapes, i.e. spherical, whisker, spiral, etc.
Fig. 2(a) and (b) shows that the higher the radiation dose, the smaller the particle size. Irradiation of Cs-colloid seems to bring the particle size down to lower than that of Cs-flake. The particle sizes are down to less than 100 nm, i.e. 72712 nm, when using g-ray dose as low as 5 kGy. The variations in particle shape and size were observed in Chi-acid condition. Our observation implied that the Cs-colloid would serve as a proper physical formulation in preparing chitosan nanoparticle via gamma irradiation.

FIG. 1. TEM images and size distribution of chitosan nanoparticle from 10 kGy gamma irradiation to (a) Cs-flake and (b) Cs-colloid (Pasanphan et al., 2010).

FIG. 2. Relationship between particle size and gamma radiation dose of (a) Cs-flake and Cs-colloid and (b) Cs-acid (Pasanphan et al., 2010).
2.2. Preparation of deoxycholate-chitosan nanoparticle (DC-Cs): chemical conjugation of DC onto gamma irradiated chitosan

To formulate the hydrophobic core to irradiated chitosan, deoxycholic acid (DC) was chemically conjugated onto irradiated Cs-flake and Cs-colloid. The preparation was carried out in a heterogeneous system according to our previous report [14]. Chitosan nanoparticles obtained in the previous preparation were in the formulation of colloid. We suspected that the DC could be conjugated on the surface of the colloidal particle.

![Graph A](image1.png) ![Graph B](image2.png)

**FIG. 3.** (A) Particle size of (a) irradiated Cs-flake, (b) irradiated Cs-flake after conjugated with DC, (c) irradiated Cs-colloid, (d) irradiated Cs-colloid after conjugated with DC. (B) Particle size of (a) Cs-colloid, (b) DC-Cs from heterogeneous, and (c) DC-Cs from homogeneous reactions [13, 15].

In the aqueous hydrophilic system, the self-assembly formulation occurred when the hydrophobic conjugated-DC side groups turned into the interior particle to serve as hydrophobic core. Fig. 3(A) indicated that the pre-irradiation dose, physical formulation of chitosan, and DC conjugation influences to the size of DC-Cs nanoparticle. The average sizes of DC-Cs were observed to be as small as 46 nm for Cs-colloid (Fig. 3d) and 72 nm for Chi-flake (Fig. 3b) when DC was conjugated onto 10 kGy gamma pre-irradiated chitosan in Fig. 3(c) and (a), respectively. Fig. 3(B) shows that the 10 kGy pre-irradiation could reduce the particle size of DC-Cs when conjugating under simple heterogeneous reaction, which only EDC conjugating agent was used. Pre-irradiation before DC-conjugation would be alternative protocol instead of the homogenous reaction, which the EDC/NHS system under several steps of preparation should be carried out [15].

2.3. PEG modified hydrophobic-chitosan nanoparticle gamma radiation synthesis

In the previous section, the effect of pre-irradiation dose to DC-Cs particle shape and size was observed. Only hydrophobic core was prepared in such view point. Here, the radiolytic methodology in preparing the polyethylene glycol (PEG) hydrophilic-shell is proposed.
2.3.1. PEG modified deoxycholate-chitosan nanoparticle (DC-Cs-PEG)
Polyethylene glycol monomethacrylate was grafted onto the surface of DC-Cs particle under aqueous mild system using gamma irradiation with the dose of 2, 4, 6, 8, and 10 kGy (7.7 kGy/h, Co-60). TEM images in Fig.4 clearly show the PEG shell of DC-Cs-PEG particle even 2 kGy irradiation.

![TEM images](image)

**FIG. 4.** TEM images of PEG shell conjugated onto DC-Cs (DC-Cs-PEG nanoparticle) prepared from radiation synthesis with different doses of (a) 2 kGy, (b) 4 kGy, (c) 6 kGy, (d) 8 kGy, and (e) 10 kGy.

Atomic force microscope (AFM) micrograph (Fig. 5a) confirms the nanoparticle formation as expected (Fig. 5b). The image implied that the core area of DC-Cs-PEG may provide the hollow core as the morphology seem to collapse in the middle portion after drying on the mica substrate.

![AFM images](image)

**FIG. 5.** (a) AFM images and (b) schematic draw of DC-Cs-PEG nanoparticle.
Fig. 6 shows the effect of radiation dose on the %grafting of PEG onto DC-Cs and subsequently affected to hydrophilic shell thickness. The consistency of shell thickness and the % grafting was observed (Fig 6A). Particle size was approximately 80 nm in the case of 2 kGy (Fig. 6A).

![Graph showing Shell thickness (nm) vs % Grafting vs Radiation dose (kGy)](image1)

**FIG. 6.** (A) Plot of particle sized and %grafting against radiation dose of DC-Cs-PEG nanoparticle. (B) Particle, core, and shell size of DC-Cs-PEG.

2.3.2. PEG modified stearyl-chitosan nanoparticle (SMA-Cs-PEG)

The preparation of SMA-Cs-PEG was completely carried out via radiolysis reaction. Stearyl methacryllate was instead used as hydrophobic core of chitosan to form steary-chitosan (SMA-Cs). In the second step, the PEG was then be grafted onto SMA-Cs via radiolytic methodology as mention in the previous section. The comparative TEM images and size distribution plot (Fig. 7) suggested that SMA-Cs-PEG gives very small particle size approximately 40 nm with narrow size distribution (Fig. 7b) when compared to the SMA-Cs (Fig. 7a). Additionally, it was found that this preparation system gave the self-assembly chitosan nanoparticle with the size less than DC-Cs-PEG (Fig. 7c).
FIG. 7. TEM images and size distribution plot of (a) SMA-Cs, (b) SMA-Cs-PEG, and (c) DC-Cs-PEG.
3. MODIFIED CHITOSAN NANOPARTICLE AS A GREEN ADDITIVE FOR MEDICAL PLASTIC

3.1. SMA-grafted-chitosan nanoparticles (SMA-Cs) using gamma radiation synthesis
Stearyl methacrylate was grafted onto non-chitosan (Cs0) and 40 kGy pre-irradiated chitosan (Cs40) via radiolytic methodology. The Cs:SMA of 1:5 gave the highest %grafting of SMA onto Cs0 at 25 kGy (Fig. 8a) and that of 1:7 for Cs40 at 10 kGy (Fig. 8b).

The particle sizes of SMA-Cs0 and SMA-Cs40 were determined in the condition that the individual particles were formed as shown in Fig. 9. At the same radiation grafting dose the particle size of SMA-Cs40 was found to be smaller than SMA-Cs0.

3.2. Pyperidine (PPD) conjugated onto SMA-Cs nanoparticles (SMA-Cs-PPD)
The SMA-Cs0 (1:5 SMA:Cs, 25 kGy) and SMA-Cs40 (1:7 SMA:Cs, 10 kGy) was chemically conjugated with pyperidine (PPD) molecule to obtain SMA-Cs0-PPD and SMA-Cs40-PPD, respectively. Fig. 10 indicates the smaller particle size observed after SMA-Cs0 and SMA-Cs40
were conjugated with PPD. The particle size of SMA-Cs0-PPD and SMA-Cs40-PPD were approximately 85 and 60 nm.

**FIG. 10.(A) TEM images of SMA-Cs and SMA-Cs-PPD from (a) non-irradiated, Cs0 and (b) 40 kGy pre-irradiated chitosan, Cs40. (B) Particle size plot of SMA-Cs and SMA-Cs-PPD.**

### 3.3. SMA-Cs and SMA-Cs-PPD nanoparticle compounding to biomedical plastic

The SMA-Cs0-PPD and SMA-Cs40-PPD nanoparticles were compounded with polylactic acid (PLA) to observe the compatibility. It was found from SEM images that SMA-Cs0-PPD and SMA-Cs40-PPD show good compatible with PLA when comparing with non-modified chitosan and with SMA-Cs0 of the lowest grafting percentage.

**FIG. 11. SEM images of SMA-Cs and SMA-Cs-PPD nanoparticle compounding with PLA.**
4. CONCLUSIONS

The present experiments proved that radiolytic methodology is a simple and effective procedure beside chemical one to fabricate hydrophobic/hydrophilic self-assembly chitosan nanoscale particle. Hydrophobic core would provide a loading space for water-insoluble drugs, whereas the modification of hydrophilic shell affects pharmacokinetic behavior. To use gamma irradiation not only induce modification via radical reaction but also diminish chemical use as well as reduce the step of preparation.

ACKNOWLEDGEMENTS

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REFERENCES


RADIATION SYNTHESIS OF POLY(N-VINYL PYRROLIDONE) NANOGELS AND NANOSCALE GRAFTING OF POLY(ACRYLIC ACID) FROM CELLULOSE

Olgun Guven, Semiha Duygu Isik, Murat Barsbay, TURKEY

Summary

Ionizing radiation has long been known to be a very useful tool for the preparation of nanogels. Although preparation is straightforward, the control of the sizes of nanogels has been a challenging issue. This report shows the results of our work on using radiation for the synthesis of PVP nanogels in the range of 40-200nm by making use of the principles of solution thermodynamics of aqueous polymer solutions. Nanoscale grafting of responsive polymers however has been of scientific and industrial importance due to fine control of the molecular weight and molecular weight distribution of grafted polymers. The second part of this report deals with the grafting of poly(acrylic acid) onto the surface of cellulose, thus imparting pH response to the substrate. The use of radiation as a constant source of radical generation and Reversible-Addition-Fragmentation-Chain transfer agents for the control of free radical polymerization provided a full control over the molecular weight and distribution of poly(acrylic acid) grafts on cellulose.

1. INTRODUCTION

Irradiation of monomers and polymers with high energy radiation (Gamma rays, X-rays, accelerated electrons and swift heavy ions) leads to the formation of very reactive intermediates in the forms of excited states, ions and free radicals. These intermediates are almost instantaneously used up in several reaction pathways which result in chain reactions, disproportionation, hydrogen abstraction arrangements or formation of new bonds, structures. The ultimate effects of these reactions are the formation of polymers, oxidized products, crosslinking and/or scissioning of main or side chains, curing and grafting. The degree of dominance of these transformations depends on the nature of the polymer and the conditions of treatment before, during and after irradiation. Close control of these factors make the modification of polymers possible by radiation processing. Polymers are generally classified as predominantly undergoing degradation or crosslinking when exposed to ionizing radiation. These two major ultimate effects of ionizing radiation have been effectively used in polymer and plastics industries since the inception of radiation technology in early 1960s. Radiation processing has been exclusively confined to the modification of bulk properties of polymers. As the need to control the properties of materials at nanoscale has moved from laboratory studies into industrial applications and nanotechnology has become an important industrial reality, the ionizing radiation has been considered for nanostructuring of polymers. Radiation can be used both for top-down and bottom-up approaches for the preparation of nanomaterials. In this report two cases of bottom-up syntheses of nanostructures will be discussed, the first one is based on the radiation-induced crosslinking of poly(vinyl pyrrolidone) in its dilute aqueous solutions for the formation of nanosized gels and the other is controlled grafting of poly(acrylic acid) from cellulose surfaces.

The preparation of nanogels using ionizing radiation has been initiated by the classical works of Rosiak and Ulanski almost two decades ago. When predominantly crosslinking type of water soluble polymers are irradiated with gamma rays or accelerated electrons, intramolecularly crosslinked polymer coils are obtained in dilute solutions. Dilute solutions are required to avoid the formation of microgels or wall-to-wall gels. The individual polymer coils thus have been converted
into networks which are termed as nanogels. In similar studies of radiation-synthesis of nanogels the molecular weight of starting polymers is the determining factor for the final size of the nanogels since coil dimensions depend on molecular weights. The control of sizes of nanogels has therefore been a challenging issue and limited with the initial molecular weight of the polymer. In dilute aqueous polymer solutions however the coil sizes of polymer chains can be controlled around the Theta/Flory temperatures. At this particular temperature is approached the coils start to contract with eventual collapse at the Theta temperature resulting with precipitation. Here in this work, for the first time we used this approach and irradiated PVP solutions in acetone/water mixtures around Theta temperature to obtain nanogels of certain size. As for the second part of our work, we combined γ-radiation and Reversible-Addition-Fragmentation-Chain Transfer (RAFT) polymerization for the preparation of graft copolymers with well-defined and smart surface properties. Renewable and natural based intelligent cellulosic surfaces that respond to pH have been prepared via this facile and powerful combination. In first year of this CRP, we reported on the preparation of novel thermo-responsive cellulose surfaces of poly(N-isopropylacrylamide) (PNIPAAm) by using the same approach and in this report we discuss the preparation of pH-responsive cellulose surfaces by grafting of poly(acrylic acid) (PAA) via γ-initiated RAFT graft polymerization technique. The response of PAA grafted surfaces to changing pH values was investigated by following the changes in the contact angle of water.

2. EXPERIMENTAL PART

2.1. Synthesis of poly(N-vinyl pyrrolidone) nanogels

Poly (vinyl pyrrolidone) (BASF, $M_w = (1.278 \pm 0.023) \times 10^6$ g mol$^{-1}$ determined by static light scattering) was used as received without further purification. Acetone (Sigma-Aldrich, Chromasolve® for HPLC, 99.9%) is used without further purification and all the solutions were prepared with deionized water with a max conductivity of 0.01 μS and filtered through 0.2-μm-pore-size Durapore filters (Millipore Corp.) prior to experiments. Poly(vinyl pyrrolidone) nanogels were synthesized via radiation induced crosslinking method in the presence and absence of acetone in aqueous solutions of PVP. All solutions were prepared freshly and placed in glass vials sealed with rubber septa and saturated with N$_2$O for 10 min prior to irradiation. Dionized water and HPLC grade solvents were used for sample preparation and analysis.

Gamma irradiations were carried out at Sarayköy Nuclear Research and Training Center, Ankara by placing the samples inside the irradiation chamber of a Gamma Cell (Tenex-Issledovatel) at ambient temperature. A $^{60}$Co source with an average dose rate of 1.34 kGy/h was used for the irradiation. Samples were taken from the chamber at different time intervals to adjust the total absorbed dose as 5, 10 and 15 kGy.

The nanogels were characterized by using nanosizer, AFM as well as SEM techniques.

2.2. RAFT-mediated grafting of PAA from cellulose

The monomer, Acrylic Acid (BDH) after standard purification step was dissolved with the RAFT agent BPATT in deionized water-ethanol mixture (9:1 v/v). The monomer concentrations and the monomer/chain transfer agent (CTA) ratios are given in Table 1. After complete dissolution of the reactants, the stock solution was divided into 10 mL aliquots and transferred to glass sample vials. BPATT-immobilized cellulose, i.e. macro-CTA, (≥0.01 g) was also added to vials as the substrate to be grafted. The vials were capped with rubber septa and deoxygenated by purging with nitrogen
gas for 20 min each. The samples were gamma irradiated with a $^{60}$Co source at ambient temperature, and then removed periodically to determine conversion and relevant properties of the synthesized polymers. Monomer to polymer conversions was evaluated using $^1$H NMR spectroscopy. Synthesized cellulosic copolymers were purified with sufficient rinsing. Details of this purification and calculation method for graft ratio ($G.R.$, wt.%) and graft frequency ($G.F.$) were described elsewhere[6].

GPC characterization was performed in DMAc (0.03% w/v LiBr, 0.05% BHT) at 40 °C (flow rate 1mL min$^{-1}$). Contact angle (CA) measurements were achieved using a Krüss DSA100 model CA goniometer. Drop volumes were 10 µL and the average CA value was obtained by measuring the same sample in four different positions. XPS measurements were carried out on a VG ESCALAB220i-XL surface analysis instrument with a mono-chromatized Al Kα X-ray source.

3. RESULTS AND DISCUSSION

3.1. Synthesis of poly(N-vinyl pyrrolidone) nanogels

Dynamic Light Scattering Analysis

Table 1 shows the peak mean diameters, their standard deviations and polydispersity index values (PDI) for nanogels that were synthesized from 1 mg/mL and 2 mg/mL aqueous PVP solutions by using gamma radiation whereas Table 2 shows the mentioned values for 1 mg/mL and 2 mg/mL aqueous PVP solutions which were synthesized by electron beam irradiation. It can be clearly seen that 2 mg/mL concentration is rather high for this synthesis parameters both for gamma and e-beam irradiations. Especially for gamma, there is a high increase in particle size due to the formation of intermolecular crosslinks. The diameters as high as 248 nm and high PDI values support this approach where the size broadening may be a result of the combination of two processes intra- and intermolecular crosslinking occurring concomitantly. Additionally, the results were found to be very striking that 1 mg/mL gamma irradiated samples show no sensitivity to total absorbed dose which was also the same for the samples prepared in acetone/water mixtures. On the other hand e-beam irradiated samples show a substantial decrease from 59 to 46 nm as the total absorbed dose is increased to 15 kGy. These tabulated results are also collected in Figure 1 and 2 for the samples prepared by gamma radiation.
Figure 1. Effect of total absorbed dose on the peak mean diameter of PVP nanogels that are synthesized from 1 mg/mL aqueous PVP solutions by gamma radiation.

Figure 2. Effect of total absorbed dose on the peak mean diameter of PVP nanogels that are synthesized from 2 mg/mL aqueous PVP solutions by gamma radiation.
Table 1. Peak mean diameters, their standard deviations and PDI values for nanogels that are synthesized from 1 mg/mL and 2 mg/mL aqueous PVP solutions by using gamma radiation.

<table>
<thead>
<tr>
<th>gamma</th>
<th>unirradiated</th>
<th>5 kGy</th>
<th>10 kGy</th>
<th>15 kGy</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/mL</td>
<td>58.93 ± 0.75</td>
<td>76.84 ± 0.42</td>
<td>77.16 ± 0.26</td>
<td>77.00 ± 0.90</td>
</tr>
<tr>
<td>2 mg/mL</td>
<td>63.29 ± 0.37</td>
<td>206.60 ± 4.78</td>
<td>247.90 ± 5.66</td>
<td>236.40 ± 1.06</td>
</tr>
</tbody>
</table>

Table 2. Peak mean diameters, their standard deviations and PDI values for nanogels that are synthesized from 1 mg/mL and 2 mg/mL aqueous PVP solutions by using e-beam radiation.

<table>
<thead>
<tr>
<th>e-beam</th>
<th>unirradiated</th>
<th>5 kGy</th>
<th>10 kGy</th>
<th>20 kGy</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/mL</td>
<td>58.93 ± 0.75</td>
<td>52.70 ± 1.54</td>
<td>48.04 ± 0.38</td>
<td>46.35 ± 0.72</td>
</tr>
<tr>
<td>2 mg/mL</td>
<td>63.29 ± 0.37</td>
<td>151.50 ± 1.41</td>
<td>85.86 ± 1.52</td>
<td>78.64 ± 0.59</td>
</tr>
</tbody>
</table>

Figure 3 shows the mean particle diameters of PVP nanogels obtained from 2 mg/mL acetone/water mixtures by means of gamma irradiation. As the amount of non-solvent increases polymer-polymer interactions are increased and the contraction of coils help the system prefer intramolecular crosslinking due to shorter inter-radical distances on the same PVP chain. Additionally, Figure 3 also expresses these results in comparison with pristine PVP. A decrease from 236 nm to 44 nm is a clear evidence that this is an efficient and useful approach to control the size of PVP nanogels.

![Figure 3](image_url)
Size distribution of unirradiated PVP coils and PVP nanogels obtained from irradiated Acetone/water solutions are shown in Figure 4. The broad distribution observed for the pristine PVP is seen to become narrower with increasing extent of intramolecular crosslinking upon formation of the nanogels.

Figure 4. Size distribution graph based on scattered light intensities of unirradiated PVP (blue) and PVP nanogels prepared in acetone/water mixtures with acetone ratios 0.60 (red), 0.62 (black), 0.64 (pink) and 0.66 (green) from 2 mg/mL PVP solutions using gamma rays with a total absorbed dose of 15 kGy.

Figure 5 shows the 3D views of the AFM images of mica surface, and soluble PVP and PVP nanogel deposited mica surfaces. The change in the surface profile after the deposition of linear PVP is clearly seen where linear PVP chains coated the surface as a film. However, the background surface of mica in Figure 5-A and PVP deposited mica in Figure 5-C remains to be unchanged which is a proof of the presence of crosslinking. For 5-C small spikes arising from the deposited nanogels are clearly seen. The roughness values are 5.63 nm for mica surface, 4.91 nm for PVP coated mica surface and 12.6 nm for PVP nanogels deposited on mica surface. For better identification of PVP nanogels, cross-sectional views were also taken from previous AFM images. Nanogels deposited on mica surface were found to be of equal size and dimensions, with approximate diameters of 80 nm.
3.2. Synthesis of RAFT-grafted PAA from cellulose

Table 3 and Figure 5 and 6 summarize the results of homo polymers formed during the graft polymerization of AA from cellulose substrate under γ-irradiation at room temperature in aqueous media.

Table 3. Reversible addition fragmentation chain transfer (RAFT) graft polymerization of AA by γ-initiation (0.02 kGy·h⁻¹) with BPATT as the RAFT agent

<table>
<thead>
<tr>
<th>Entry</th>
<th>Monomer</th>
<th>([M]<em>b) (\text{[CTA]}</em>{hi})</th>
<th>time (min)</th>
<th>Convn(^b) (%)</th>
<th>G.R.(^c) wt.%</th>
<th>G.F.(^c)</th>
<th>M(_{n,\text{theor}})(^c) (g mol⁻¹)</th>
<th>M(_{n,\text{SEC}})(^d) (g mol⁻¹)</th>
<th>PDI(^d)</th>
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<tbody>
<tr>
<td>1</td>
<td>AA</td>
<td>285</td>
<td>390</td>
<td>21</td>
<td>&lt;2</td>
<td>1.14</td>
<td>4 590</td>
<td>8 800</td>
<td>1.24</td>
</tr>
<tr>
<td>2</td>
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<td>630</td>
<td>47</td>
<td>5</td>
<td>1.97</td>
<td>9 920</td>
<td>13 300</td>
<td>1.12</td>
</tr>
<tr>
<td>3</td>
<td>AA</td>
<td>285</td>
<td>750</td>
<td>51</td>
<td>6</td>
<td>2.14</td>
<td>10 740</td>
<td>14 100</td>
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<td>4</td>
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<td>990</td>
<td>73</td>
<td>9</td>
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<td>15 260</td>
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<td>93</td>
<td>14</td>
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<td>23 100</td>
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<td>&gt;98</td>
<td>8</td>
<td>0.11</td>
<td>-</td>
<td>397 200</td>
<td>2.62</td>
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</table>

Figure 5. 3D views of AFM images of mica surface (A), PVP coated mica surface (B), PVP nanogels deposited on mica surface (C).
RAFT graft-polymerization of [AA]$_0$=2 mol L$^{-1}$ from BPATT functionalized cellulose ($\approx$0.01 g) initiated via $\gamma$-irradiation (0.02 kGy h$^{-1}$) in water-EtOH (9:1 v/v) at room temperature. Monomer conversion was determined from NMR analysis. See ref. [15] for the details of $G.R$, $G.F.$, and the theoretical number-average molecular weight, $M_{n,\text{theor}}$. Calculations. Number-average molecular weight, $M_n$, and polydispersity indices, PDI, determined via size-exclusion chromatography, SEC, using DMAc as eluent with polystyrene standards for the non-grafted polymers formed during grafting. The filter paper was not modified with BPATT but subjected to polymerization conditions and no free BPATT was added to the medium.

As can be seen from Table 3, the apparent number-average molecular weights, $M_n$, obtained using polystyrene standards are almost comparable to the theoretical $M_n$ values of PAA. The polydispersity indices (PDI) of the resulting polymers are narrow, i.e., PDI $\leq$ 1.26, indicating a well-controlled polymerization occurred via the RAFT process. The difference between the theoretical and the experimental molecular weights can be attributed to the calibration of the GPC on the basis of polystyrene equivalents, universal calibration. In addition to the RAFT-mediated graft polymerizations, conventional graft-polymerizations were also achieved (denoted as blank in Table 3) and comparison of the results clearly confirms the controlled fashion and success of the graftings mediated via RAFT (compare the $M_n$, PDI and $G.F.$ values). As can be seen from Figure 5a the GPC traces are unimodal and narrow at all conversions.

Moreover, it is obvious that the molecular weight increases linearly with conversion (see Figure 5b where the number-average molecular weight, $M_n$, and the PDI evolutions with the conversion are depicted) which again demonstrates the well-defined behavior of the grafting for AA under the mentioned reaction conditions.

XPS was used to confirm the grafting of AA from cellulose. The surface chemical compositions calculated using the peak areas of the XPS survey wide scans indicate significant changes following the grafting: the C atom amount increases from 62.8% to 69.0% whereas the O atom amount decreases from 34.1% to 30.7% for the copolymers of PAA, respectively (see the quantification results inserted to the C1s XPS spectra in Figure 5A, C). The C1s XPS spectra show a considerable change in carbon atom amounts in different functional groups: the C1s spectrum of BPATT-immobilized cellulose (Figure 6A) consists of a main peak with a bonding energy (BE) of 286.5 eV attributed to C-O bonds. However after the grafting of AA, the main peaks appear at 285
eV, attributed to non-oxygenated C, i.e. C-C, C-H bonds. These results confirm the successful grafting of PAA from cellulose.

Figure 5. C 1s XPS spectra of (A) BPATT-immobilized cellulose; (C) cellulose-g-PAA copolymer with 14% graft ratio. The percentages inserted to C 1s spectra show the surface chemical compositions calculated using the peak areas of the XPS survey wide scans.

Responsive behavior of graft polymers
PAA responds to changes in pH and ionic strength by changing coil dimensions and solubility. In general, PAA displays a broad pKa value of 4–5 and thus a proportion of its side chain carboxyls are ionized around pH 5–6. Below this pH value, a PAA-grafted surface is hydrophobic with collapsed polymer brushes whereas it becomes hydrophilic in neutral and alkaline aqueous media.

Figure 6. Contact angle of PAA grafted cellulose surface at (A) pH=11, (B) pH=3.

The response of 14% PAA-grafted cellulosic copolymer to change in pH was characterized by static CA measurements at pH 3 and 11. The collapse of the brushes in acid media (i.e. pH 3) was reflected by changes in the wettability of the surface: at pH 11, the ionized PAA-modified cellulose surface is hydrophilic, and the applied water droplet is rapidly adsorbed into the surface within a couple of seconds (the CA measured at the third second was 28.6° ±2.4). At pH 3, well below the pKa for PAA, the cellulose surface presents an increased hydrophobic character with a CA of 68.7° ±4.5 due to the collapse of the polymer brushes. The representative figure for this phenomenon is shown on Figure 6.
Conclusions
We have shown that by considering the fundamental aspects of polymer aqueous solution thermodynamics to control the sizes of polymer coils it is possible to convert these soluble chains into intramolecularly crosslinked network structures by gamma irradiation or EB treatment. PVP nanogels of different sizes with rather low size distributions were thus synthesized in the range of 40-240nm. It has also been shown that by combination of γ-radiation and RAFT polymerization graft copolymers with well-defined and smart surface properties can be easily prepared. Renewable and natural based intelligent cellulosic surfaces that respond to pH have been prepared for the first time via this facile and powerful combination.

REFERENCES

NANOSCALE RADIATION ENGINEERING OF ADVANCED MATERIALS FOR POTENTIAL BIOMEDICAL APPLICATIONS

Allan S. Hoffman; USA

Summary

We are using RAFT polymerization to synthesize smart polymer nanocarriers for intracellular delivery of protein, peptide and nucleic acid drugs. In the coming program period we plan to synthesize these carriers using radiation to initiate the RAFT polymerizations. In this way we will avoid the need to add free radical initiators to initiate this polymerization, yielding a purer polymer-drug nanocarrier.

Achievements

Nanocarriers

Smart T- and pH-responsive polymer nanocarriers have been RAFT synthesized for enhanced intracellular delivery of biomolecular drugs such as peptides, proteins and nucleic acid drugs.

1. INTRODUCTION

The gene-knockdown activities of small, interfering RNA (siRNA) have led to their use as drug target validation tools in drug discovery, and also as potential therapeutics for a variety of diseases. The efficient intracellular delivery of these double-stranded RNA macromolecules has proven to be challenging, and achieving efficient and safe delivery of siRNA is a significant barrier to its development as a clinical therapy [1-4]. Carriers for siRNA delivery usually consist of cationic polymers, peptides or lipids that form complexes with the nucleic acid, protecting it from nuclease attack, and facilitating cell uptake through electrostatic interactions with negatively-charged phospholipid bilayers or through specific targeting moieties [5-13]. A variety of synthetically and biologically-derived polymers have been investigated for use as nucleic acid carriers including poly(dimethylaminoethyl methacrylate) (pDMAEMA) [14-17], poly(L-lysine) [18-23], polyethylenimine (PEI) [24-29], and chitosan [30-32]. While many cationic polymers are highly efficient at nucleic acid delivery, significant cytotoxicity is often observed [33-35]. In addition, anionic serum proteins can interact with net positively-charged siRNA/polycation complexes and cause aggregation or decomplexation, significantly reducing or ablating siRNA efficacy [36].

Once siRNA is endocytosed, the predominant fate is enzymatic degradation in the lysosome or recycling and extracellular clearance [37]. In order to circumvent this fate, several strategies have been employed to enhance endosomal escape. pH responsive lipid or lipid-like molecules and viral fusogenic proteins and peptides promote endosomal escape by becoming membrane destabilizing through a pH-dependent shift in their conformation [5, 8-13, 38-40]. In an effort to mimic viral endosomal escape mechanisms that trigger membrane destabilization at acidic pH, polymers that possess pH-sensitive chemical functionalities, such as carboxylate groups, have been explored [41-45]. Poly(propylacrylic acid) (PPAA) undergoes a hydrophilic-to-hydrophobic transition at endosomal pHs, mediating membrane disruption [46]. This conformational shift is triggered by the gradual protonation of carboxylic acid residues along the polymer backbone and can be tuned to occur at specific pHs by copolymerization with hydrophobic monomers [47].
The modular design of diblock polymers allows the incorporation in one block of cationic segments that complex nucleic acids and the incorporation of other segments that become membrane disruptive at endosomal pH values. Diblock polymers have been widely explored as materials as nucleic acid delivery carriers [48-53]. The synthesis of these materials was simplified with the advent of controlled radical polymerization (CRP) techniques, including reversible addition-fragmentation chain transfer (RAFT) polymerization [54-56]. These new polymerization techniques enable precise control over molecular weight polydispersities, while eliminating the need for stringent reaction conditions, and expand the scope of monomer components. A variety of compositions have been investigated for the respective block segments. However, neutral hydrophilic monomers such as poly(ethylene glycol) (PEG) and hydroxypropyl methacrylamide (HPMA) are most often chosen as stabilizing blocks because of their water solubility and low toxicity [57-60]. In addition, Zhao et al. recently reported the synthesis of block copolymers stabilized by inclusion of a zwitterionic block. This system, consisting of 2-(methacyrloyloxy)-ethylphosphorylcholine and 2-(diethylamino)-ethyl methacrylate, was shown to efficiently deliver antisense oligodeoxynucleotide to human cervical carcinoma cells [61].

We have developed a new diblock copolymer family that was designed to enhance the systemic and intracellular delivery of siRNA. These diblock copolymers were synthesized using the controlled Reversible Addition Fragmentation chain Transfer polymerization (RAFT) method, which usually employs a free radical initiator such as AIBN (Azo-bis-Isobutyryl-Nitrile); we will replace such initiators with low dose rate irradiation in future collaborations within this CRP.

The diblock polymers are composed of a positively-charged block of dimethylaminoethyl methacrylate (DMAEMA) to mediate siRNA condensation, and a second endosomal-releasing block composed of DMAEMA and propylacrylic acid (PAA) in roughly equimolar ratios, together with butyl methacrylate (BMA). A related series of diblock compositions were characterized, with the cationic first block kept at constant MW of 9100, while in the second block, the ratio of DMAEMA and PAA to BMA was varied, along with the MW. As the percentage of BMA in the second block was systematically increased, these carriers became sharply hemolytic at endosomal pH regimes.

The diblock copolymers condensed siRNA into 80-250 nm particles with slight positive zeta potentials. The siRNA knockdown activities in HeLa cells generally followed the hemolytic activity trends, with the most hydrophobic second block (highest BMA mole fraction) exhibiting the best polyplex properties and knockdown activities. This pH-responsive ampholytic carrier designed to mediate endosomal release thus shows significant promise for the intracellular delivery of siRNA.

The ability of small interfering RNA (siRNA) to efficiently silence the expression of specific genes provides the basis for exciting new therapies based on RNA interference (RNAi). The efficient intracellular delivery of siRNA, starting with cell uptake, continuing through the endosomal trafficking pathway and eventually into the cytoplasm remains a significant challenge.

2. MATERIALS AND METHODS
Materials. Chemicals and all materials were supplied by Sigma-Aldrich unless otherwise specified.

Synthesis of RAFT chain transfer agent. The synthesis of the chain transfer agent (CTA), 4-Cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT), utilized for the following RAFT polymerizations, was adapted from a procedure by Moad et al. [62]. Briefly, ethane thiol (4.72 g, 76 mmol) was added over 10 min to a stirred suspension of sodium hydride (60% in oil) (3.15 g, 79 mmol) in diethyl ether (150 ml) at 0 °C. The solution was then allowed to stir for 10 min prior to the addition of carbon disulfide (6.0 g, 79 mmol). Crude sodium S-ethyl trithiocarbonate (7.85 g, 0.049 mol) was collected by filtration, suspended in diethyl ether (100 mL), and reacted with Iodine (6.3 g, 0.025 mol). After 1 h the solution was filtered, washed with aqueous sodium thiosulfate, and dried over sodium sulfate. The crude bis (ethylsulfanylthiocarbonyl) disulfide was then isolated by rotary evaporation. A solution of bis-(ethylsulfanylthiocarbonyl) disulfide (1.37 g, 0.005 mol) and 4,4'-azobis(4-cyanopentanoic acid) (2.10 g, 0.0075 mol) in ethyl acetate (50 mL) was heated at reflux for 18 h. Following rotary evaporation of the solvent, the crude 4-Cyano-4 (ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT) was isolated by column chromatography using silica gel as the stationary phase and 50:50 ethyl acetate hexane as the eluent. \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.36 t (SCH\(_2\)CH\(_3\)); \(\delta\) 1.88 s (CCNC\(_2\)H\(_3\)); \(\delta\) 2.3-2.65 m (CH\(_2\)CH\(_2\)); \(\delta\) 3.35 q (SCH\(_2\)CH\(_3\)).

Synthesis of poly(dimethylaminoethyl methacrylate) macro chain transfer agent (pDMAEMA macroCTA). The RAFT polymerization of DMAEMA was conducted in DMF at 30 °C under a nitrogen atmosphere for 12 hours using ECT and 2,2'-Azobis(4-methoxy-2,4-dimethyl valeronitrile) (V-70) (Wako chemicals) as the radical initiator. The initial monomer to CTA ratio ([CTA]\(_0\)/[M]\(_0\)) was such that the theoretical \(M_n\) at 100% conversion was 10,000 (g/mol). The initial CTA to initiator ratio ([CTA]\(_o\)/[I]\(_o\)) was 10 to 1. The resultant pDMAEMA macro chain transfer agent was isolated by precipitation into 50:50 v:v diethyl ether/pentane. The resultant polymer was redissolved in acetonitrile and subsequently precipitated into pentane (x3) and dried overnight in vacuo.

Block copolymerization of DMAEMA, PAA, and BMA from a pDMAEMA macroCTA. (see Table 1 and Scheme 1 below) The desired stoichiometric quantities of DMAEMA, PAA, and BMA were added to pDMAEMA macroCTA dissolved in N,N-dimethylformamide (25 wt % monomer and macroCTA to solvent). For all polymerizations [M]\(_o\)/[CTA]\(_o\) and [CTA]\(_o\)/[I]\(_o\) were 250:1 and 10:1 respectively. Following the addition of V70 the solutions were purged with nitrogen for 30 min and allowed to react at 30 °C for 18 h. The resultant diblock copolymers were isolated by precipitation into 50:50 v:v diethyl ether/pentane. The precipitated polymers were then redissolved in acetonitrile and subsequently precipitated into pentane (x3) and dried overnight in vacuo. Gel permeation chromatography (GPC) was used to determine molecular weights and polydispersities (PDI, \(M_w/M_a\)) of both the poly(DMAEMA) macroCTA and diblock copolymer samples in DMF with respect to polymethyl methacrylate standards (SEC Tosoh TSK-GEL R-3000 and R-4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to a Viscotek GPCmax VE2001 and refractometer VE3580 (Viscotek, Houston, TX). HPLC-grade DMF containing 0.1 wt % LiBr was used as the mobile phase).

siRNA/polymer complex characterization. After verification of complete, serum-stable siRNA complexation via agarose gel retardation (see Supplementary Information), siRNA/polymer
complexes were characterized for size and zeta potential using a ZetaPALS detector (Brookhaven Instruments Corporation, Holtsville, NY, 15 mW laser, incident beam = 676 nm). Briefly, polymer was formulated at concentrations of 0.1-10 mg/ml in Dulbecco’s Phosphate Buffered Saline (PBS, without calcium and magnesium, Gibco) and complexes were formed by addition of polymer to GAPDH siRNA (50 μM, Qiagen, Hs_GAPDH_3 HP, sense: 5’-GGUCGGAGUCAACGGAUUU-3’, antisense: 5’AAAUCCGUUGACUCCGACC-3’). The theoretical charge ratios (+/-) are calculated using only the positively charged DMAEMA block and the negatively charged siRNA, as the zwitterionic second block is approximately 50% protonated at pH=7.4 and the ratio of DMAEMA to PAA is generally within error of 1:1. In general, the appropriate volume of siRNA was added to a tube and diluted in PBS to a concentration at 6-10x of the intended testing concentration (for particle size and Zeta measurements, the final siRNA concentration was 25 nM). The required volume of polymer was then added to bring the total complex concentration to ~5x. Particles were allowed to condense for 20 min at room temperature then were diluted in PBS to 1x and measured. Correlation functions were collected at a scattering angle of 90°, and particle sizes were calculated using the viscosity and refractive index of water at 25 °C. Particle sizes are expressed as effective diameters assuming a log-normal distribution. Average electrophoretic mobilities were measured at 25 °C using the ZetaPALS zeta potential analysis software, and zeta potentials were calculated using the Smoluchowsky model for aqueous suspensions.

HeLa cell culture. HeLa cells, human cervical carcinoma cells (ATCC CCL-2), were maintained in minimum essential media (MEM) containing L-glutamine (Gibco), 1% penicillin-streptomycin (Gibco), and 10% fetal bovine serum (FBS, Invitrogen) at 37 °C and 5% CO₂.

pH-dependent membrane disruption of carriers and siRNA/polymer complexes. Hemolysis [38, 42] was used to determine the potential endosomolytic activity of both free polymer and siRNA/polymer conjugates at pH values that mimic endosomal trafficking (extracellular pH = 7.4, early endosome pH = 6.6, and late endosome pH = 5.8). Briefly, whole human blood was collected in vacutainers containing EDTA. Blood was centrifuged, plasma aspirated, and washed three times in 150 mM NaCl to isolate the red blood cells (RBC). RBC were then resuspended in phosphate buffer (PB) at pH 7.4, pH 6.6, or pH 5.8. Polymers (10 μg/ml) or polymer/siRNA complexes were then incubated with the RBC at the three pH values for 1 hour at 37 °C. Intact RBC were then centrifuged and the hemoglobin released into supernatant was measured by absorbance at 541 nm as an indication of pH-dependent RBC membrane lysis.

Measurement of carrier-mediated siRNA uptake. Intracellular uptake of siRNA/polymer complexes was measured using flow cytometry (Becton Dickinson LSR benchtop analyzer). HeLa cells were seeded at 15,000 cells/cm² (6-well plates) and allowed to adhere overnight. FAM labeled siRNA (Ambion) was complexed with polymer at a theoretical charge ratio of 4:1 for 30 min at room temperature and then added to the plated HeLa at a final siRNA concentration of 25 nM (1000 μl volume). After incubation with the complexes for 4 h, the cells were trypsinsized and resuspended in PBS with 0.5% BSA and 0.01% trypan blue. Trypan blue was utilized as previously described for quenching of extracellular fluorescence and discrimination of complexes that have been endocytosed by cells [63]. 10,000 cells were analyzed per sample and fluorescence gating was determined using samples receiving no treatment and polymer not complexed with FAM-labeled siRNA (Ambion Negative Control #1, FAM-labeled).
siRNA/polymer complex cytotoxicity. siRNA/polymer complex cytotoxicity was determined using and lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche). HeLas were seeded in 96-well plates at a density of 12,000 cells/cm² and allowed to adhere overnight. Complexes were formed by addition of polymer (0.1 mg/ml stock solutions) to GAPDH siRNA at theoretical charge ratios of 4:1 and to attain a concentration of 25 nM siRNA/well (100 µL volume). Complexes (charge ratio = 4:1) were added to wells in triplicate. After cells had been incubated for 24 h with the polymer complexes, the media was removed and the cells were washed with PBS twice. The cells were then lysed with lysis buffer (100 µL/well, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) for 1 hour at 4 °C. After mixing by pipetting, 20 µL of the cell lysate was diluted 1:5 in PBS and quantified for lactate dehydrogenase (LDH) by mixing with 100 µL of the LDH substrate solution. After 10-20 min incubation for color formation, the absorbance was measured at 490 nm with the reference set at 650 nm.

Evaluation of GAPDH protein and gene knockdown by siRNA/polymer complexes. The efficacy of the series of polymers for siRNA delivery was screened using a GAPDH activity assay (Ambion). HeLas (12,000 cells/cm²) were plated in 96-well plates. After 24 h, complexes (charge ratios = 4:1) were added to the cells at a final siRNA concentration of 25 nM in the presence of 10% serum (100 µL volume). The extent of siRNA-mediated GAPDH protein reduction was assessed 48 h post-transfection. As a positive control, parallel knockdown experiments were run using HiPerFect (Qiagen) following manufacturer’s conditions. The remaining GAPDH activity was measured as described by the manufacturer using the kinetic fluorescence increase method over 5 min and was calculated according to the following equation: % remaining expression = ∆fluorescence, GAPDH/∆fluorescence, no treatment where ∆fluorescence = fluorescence₅min−fluorescence₁min.

After the initial screen to identify the carrier that produced the most robust siRNA-mediated GAPDH knockdown, real time reverse transcription polymerase chain reaction (RT-PCR) was used to directly evaluate siRNA delivery. After 48 hours of incubation with complexes as formed above, cells were rinsed with PBS. Total RNA was isolated using Qiagen’s Qiashredder and RNeasy mini kit. Any residual genomic DNA in the samples was digested (RNase-Free DNase Set, Qiagen) and RNA was quantified using the RiboGreen assay (Molecular Probes) based on the manufacturer’s instructions. Reverse transcription was performed using the Omniscript RT kit (Qiagen). A 25 ng total RNA sample was used for cDNA synthesis and PCR was conducted using the ABI Sequence Detection System 7000 using predesigned primer and probe sets (Assays on Demand, Applied Biosystems) for GAPDH and β-actin as the housekeeping gene. Reactions (20 µl total) consisted of 10 µL of 2X Taqman Universal PCR Mastermix, 1 µL of primer/probe, and 2 µL of cDNA, brought up to 20 µL with nuclease-free water (Ambion). The following PCR parameters were utilized: 95 °C for 90 s followed by 45 cycles of 95 °C for 30 s and 55 °C for 60 s. Comparative threshold cycle (Cₜ) analysis was used to quantify GAPDH, normalized to β-actin and relative to expression of untreated HeLas.

Statistical methods. ANOVA was used to test for treatment effects, and Tukey’s test was used for post hoc pairwise comparisons between individual treatment groups.

3. KEY RESULTS AND DISCUSSION
Endosomolytic activity of carriers and siRNA/polymer complexes. Both polymer and siRNA/polymer complexes were evaluated for their ability to induce red blood cell hemolysis at pH values relevant to the endosomal/lysosomal trafficking pathway (Figure 1). No significant hemolysis was observed for polymers 1-3. Significant pH-dependent hemolytic activity was evident first with polymer 4, and enhanced activity was found as BMA content of the endosomolytic block increased. Polymer 7 exhibited the greatest pH-dependent hemolysis with essentially no activity at pH = 7.4, about 25% hemolysis at pH = 6.6, and 85% hemolysis at pH = 5.8. Polymers 5-7 were subsequently evaluated for hemolytic activity in their siRNA-complexed form, and complexes formed with polymers 5-7 at all charge ratios tested were found to be hemolytic in a pH-dependent fashion. The hemolysis exhibited by complexes was increased when compared with free polymer and was greater at a charge ratio of 4:1 versus 1:1. Polymer 7 showed the greatest hemolytic activity at a charge ratio of 4:1, with essentially no hemolysis at pH = 7.4, 60% hemolysis at pH = 6.8, and 100% hemolysis at pH 5.8. These data indicate that the pH-responsive hemolytic activity of these polymers is tightly linked to the incorporation of a hydrophobic moiety, butyl methacrylate. This finding corroborates previous reports on pH-responsive, membrane destabilizing polymers that have utilized incorporation of hydrophobic moieties such as alkyl amines or aromatic groups to enhance the pH-dependent hydrophobic transition of carboxylate functionalized polymers [42, 47, 72].

Carrier-mediated siRNA uptake. Cellular internalization of siRNA complexes at 4:1 charge ratios was investigated using flow cytometry for polymers 4-7 based on their relevant pH-responsive endosomolytic characteristics (see Figure 2 and Table 2). As expected, all polymer formulations showed much greater uptake (up to 25x) by cells than siRNA not complexed with a carrier (naked siRNA). Cellular uptake was also found to positively correlate with BMA content of the second block, with polymer 7 showing the highest level of uptake (23% siRNA positive cells) during this timeframe (see Figure 2). Internalization of complexed siRNA by up to 23% of cells after only 4 h is a promising result, as the cumulative uptake is likely to be much higher after the full 48 h treatment. In addition, siRNA activity is considered to be catalytic; it can be recycled within the cytoplasm to destroy multiple mRNA transcripts, therefore having a long-term, multi-generational effect [73]. The smaller size of the polymer 7 complexes could be a factor in the increased internalization, together with the enhanced endosomolytic effectiveness of the BMA-containing block. We have shown recently that PAA-containing protein conjugates exhibit reduced extracellular recycling and increased accumulation of protein within the cell [74] compared to conjugates made with the analogous non-destabilizing monomers. The combination of increased uptake and endosomal release leads to strongly enhanced intracellular concentrations of siRNA with the polymer 7 complexes.

siRNA/polymer complex cytotoxicity. The cytotoxicity of the polymer carriers was investigated by incubating HeLa cells in the presence of the complexes at charge ratios of 4:1 for 24 h. The resulting cell survival, as measured by intracellular lactate dehydrogenase activity versus untreated cells, showed that high relative survival was observed (>90% after 24 h) for all polymers tested. Synthetic polymers, in particular cationic polymers, can be associated with appreciable cytotoxicity. For instance, PEI has been shown to trigger apoptosis and/or necrosis in a variety of cell lines [75]. This toxicity can be reduced by chemically modifying the polycation segment with hydrophilic segments [76], however, there is usually a tradeoff between efficacy and toxicity [77].
In this approach, the use of a near-neutral polyampholyte for the second block of the polymer delivery vehicle reduced the intrinsic cytotoxicity of the polycation block with the cultured HeLas.

Evaluation of GAPDH protein and gene knockdown by siRNA/polymer complexes. The ability of the carriers to effectively deliver siRNA was investigated in knockdown experiments against GAPDH with complexes formed from all polymers at theoretical charge ratios of 4:1. GAPDH protein levels were evaluated 48 h after treatment with the complexes, and data are shown relative to GAPDH protein levels of untreated cells (Figure 2, black bars). Polymer carriers 1-3 were ineffectual at eliciting reduction of protein levels, likely due to their inability to mediate endosomal escape. However, GAPDH protein reduction became evident with the use of polymer 4 as a siRNA carrier. The knockdown of protein further increased as the BMA content of the carriers increased to 48% of the endosomolytic block (polymer 7). Polymer 7 showed the greatest ability to mediate siRNA knockdown of protein where GAPDH was reduced to 32% of control.

To further characterize carrier efficacy, polymers were analyzed for their ability to knockdown GAPDH mRNA levels. Similar to the protein measurements, polymers 1-3 elicited very little reduction of mRNA signal, as evaluated by RT-PCR (Fig.2). Again, polymers 4-7 showed increased knockdown of GAPDH as the BMA content of the endosomolytic block increased. Specifically, GAPDH knockdown was reduced to 39%, 30%, 31%, and 21% of control at a charge ratio of 4:1, for polymers 4, 5, 6, and 7, respectively. Overall, our results are consistent with findings from other groups exploring delivery strategies for DNA which have found that the addition of hydrophobic domains, specifically N-oleyl moieties, phenylalanine residues, and butyl methacrylate, as utilized here, enhance transfection [64-66].

Because the polymer 7, with the greatest butyl methacrylate content in the endosomolytic block, showed the most promise as a siRNA carrier, a further investigation into its ability to mediate gene knockdown was performed with respect to charge ratio and siRNA dose. Alteration of theoretical charge ratios was found to strongly affect gene knockdown. GAPDH was reduced to 51%, 42%, 21%, and 14% of control levels with charge ratios of 1:1, 2:1, 4:1, and 8:1, respectively. Particularly at charge ratios of 4:1 and 8:1, gene knockdown was similar to the commercially available carrier HiPerFect, where GAPDH levels were reduced by over 80%. Importantly, the effects on GAPDH levels are specific to the siRNA that is delivered, as when a control siRNA is utilized at a charge ratio of 8:1, there is no significant effect on GAPDH levels. Altering the charge ratio may have resulted in differing levels of condensation of the siRNA within the nanoparticles. Our DLS experiments indicated that increasing copolymer content in the complexes resulted in more condensed particles and these functional studies suggest that more compact particles can be internalized more efficiently or with increased siRNA bioavailability. These findings are consistent with previous reports indicating that more compact DNA/polyethyleneimine and DNA/polylysine complexes internalize at higher rates and achieve higher transfection efficiencies [78, 79].

We also completed a dose-response study using P7 at a charge ratio of 4:1. Although there was little response in GAPDH gene expression at 1 nM or 5 nM siRNA, expression was reduced to 77%, 21%, and 12% of control when 10 nM, 25 nM, or 50 nM of siRNA was delivered using polymer 7. This level of knockdown approaches that seen using 50 nM HiPerFect, a commercially available positive control. However, all the diblock copolymers demonstrated enhanced biocompatibility, as measured by cytotoxicity assays compared to HiPerFect.

We have recently developed a new generation of siRNA delivery polymers that exhibit enhanced transfection efficiency and low cytotoxicity. This design incorporates a longer endosomolytic, second block with increased hydrophobic content to induce micelle formation. These polymers
spontaneously form spherical micelles in the size range of 40 nm with CMC (critical micelle concentration) values of approximately 2 µg/ml based on dynamic light scattering (DLS), 1H-NMR, electron microscopy, and selective partitioning of the small molecule pyrene into the hydrophobic micelle core. The siRNA binding to the cationic shell block did not perturb micelle stability or significantly increase particle size. The self-assembly of the diblock copolymers into particles was shown to provide a significant enhancement in mRNA knockdown at siRNA concentrations as low as 12.5 nM. Under these conditions the micelle-based systems showed an 89% reduction in GAPDH mRNA levels as compared to only 23% (10 nM siRNA) for the non-micelle system. The reduction in mRNA levels becomes nearly quantitative as the siRNA concentration is increased to 25 nM and higher. Flow cytometry analysis of fluorescent-labeled siRNA showed uptake in 90% of the cells and a 3-fold increase in siRNA per cell compared to a “gold standard” lipid transfection agent. These results demonstrate the potential utility of this carrier design for siRNA drug delivery. See Fig. 4 below from ref. 80.

REFERENCES


Table 1. Molecular weights, polydispersities, and monomer compositions for the poly(DMAEMA)-macroCTA, the resultant diblock copolymers, and their corresponding nomenclature.

<table>
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<th>$M_2^*$</th>
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$^a$ As determined by SEC Tosoh TSK-GEL R-1000 and R-4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to a Viscocet GPCmax VE2001 and refractometer VE3580 (Viscoteck, Houston, TX). HPLC-grade DMF containing 0.1 wt % LiBr was used as the mobile phase. The molecular weights of the synthesized copolymers were determined using a series of poly(methyl methacrylate) standards.

$^b$ As determined by $^1$H NMR spectroscopy (3 wt % in CDCl₃; Bruker DRX 400).
Scheme 1. RAFT mediated synthesis of diblock copolymers consisting of a cationic poly(DMAEMA) block and an endosomolytic polyampholyte block.
FIG. 1.
FIG. 2.
Table 2. Size and ζ-potential measurements of particles formulated with siRNA at a theoretical charge ratio of 4:1 as a function of butyl methacrylate composition.

<table>
<thead>
<tr>
<th>Polymer #</th>
<th>Diameter (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>166</td>
<td>0.14</td>
<td>1.1</td>
<td>1.32</td>
</tr>
<tr>
<td>P2</td>
<td>189</td>
<td>0.09</td>
<td>0.13</td>
<td>0.69</td>
</tr>
<tr>
<td>P3</td>
<td>197</td>
<td>0.06</td>
<td>0.47</td>
<td>0.59</td>
</tr>
<tr>
<td>P4</td>
<td>144</td>
<td>0.11</td>
<td>0.41</td>
<td>1.2</td>
</tr>
<tr>
<td>P5</td>
<td>193</td>
<td>0.32</td>
<td>0.52</td>
<td>0.77</td>
</tr>
<tr>
<td>P6</td>
<td>236</td>
<td>0.06</td>
<td>0.67</td>
<td>0.95</td>
</tr>
<tr>
<td>P7</td>
<td>85</td>
<td>0.20</td>
<td>0.18</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 4. Hemolysis of the (a) diblock copolymer as a function of pH at concentrations of 2.2, 4.5, 9.0, and 18 μg/mL and (b) diblock copolymer/siRNA complexes at theoretical charge ratios of 1:1, 2:1, 4:1, and 8:1 (25 nM siRNA). Hemolytic activity is normalized relative to a positive control, 1% v/v Triton X-100, and the data represent a single experiment conducted in triplicate ± standard deviation.
## Participants List

2nd RCM on
"Nanoscale Radiation Engineering of Advanced Materials for Potential Biomedical Applications"
15-19 November 2010
Paris, France

<table>
<thead>
<tr>
<th></th>
<th>Name</th>
<th>Institution</th>
<th>Address</th>
<th>Phone</th>
<th>Fax</th>
<th>Email</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Argentina</td>
<td>Mr. Mariano Grasselli</td>
<td>Quesada 2422, piso 11, dpto. C C1429 Buenos Aires</td>
<td>+541143657100 Ext: 4346</td>
<td>+541143657132</td>
<td><a href="mailto:mgrasse@unq.edu.ar">mgrasse@unq.edu.ar</a>; <a href="mailto:mariano.grasselli@gmail.com">mariano.grasselli@gmail.com</a></td>
</tr>
<tr>
<td>2</td>
<td>Brazil</td>
<td>Mr. Ademar Benevolo Lugao</td>
<td>Comissão Nacional de Energia Nuclear (CNEN); Instituto de Pesquisas Energeticas e Nucleares (IPEN)</td>
<td>+5511313139250</td>
<td></td>
<td><a href="mailto:ablugao@ipen.br">ablugao@ipen.br</a></td>
</tr>
<tr>
<td>3</td>
<td>China</td>
<td>Mr. Qingde Chen</td>
<td>Department of Applied Chemistry College of Chemistry and Molecular Engineering Peking University</td>
<td>+861062753443</td>
<td>+861062753794</td>
<td><a href="mailto:qdchen@pku.edu.cn">qdchen@pku.edu.cn</a></td>
</tr>
<tr>
<td>4</td>
<td>Egypt</td>
<td>Mr. Hassan Ahmed AbdEl-Rehim</td>
<td>National Centre for Radiation Research Research Centre (NCRRT), Atomic Energy Authority NCRRT, P.O.Box 29, Nasr City Cairo</td>
<td>+000202-22749892</td>
<td></td>
<td><a href="mailto:ha_rehim@hotmail.com">ha_rehim@hotmail.com</a></td>
</tr>
<tr>
<td>5</td>
<td>France</td>
<td>Mr. Giancarlo Rizza</td>
<td>Commissariat à l'énergie atomique (CEA); Institut Rayonnement Matière de Saclay (IRaMIS)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 6. Hungary          | Mr. Miklos Veres  
|                    | Hungarian Academy of Sciences; Research Institute for Solid State Physics and Optics; Department of Laser Applications  
|                    | Konkoly Thege Miklós ut 29-33  
|                    | 1121 Budapest  
|                    | P.O. Box 49  
|                    | 1525 Budapest  
|                    | Tel: +3613922212  
|                    | Fax: +3613922215  
|                    | Email: vm@szfki.hu |
| 7. India           | Ms. Jayashree Biswal  
|                    | Radiation Technology Development Division  
|                    | Bhabha Atomic Research Centre  
|                    | Mumbai-400085  
|                    | Tel: +91 22 25590175, 09969613154  
|                    | Fax: +912225505338  
|                    | Email: jibiswal@barc.gov.in, jayashree_biswal@yahoo.co.in |
| 8. Iran            | Ms. Susan Dadbin  
|                    | Atomic Energy Organization of Iran (AEOI)  
|                    | Nuclear Science and Technology Research Institute  
|                    | P.O. Box 11365-8486  
|                    | Tehran, North Kargar  
|                    | Email: sdadbin@yahoo.com; sdadbin@aeoi.org.ir |
| 9. Italy           | Ms. Clelia Dispenza  
|                    | Universita degli Studi di Palermo;  
|                    | Dipartimento di Ingegneria Industriale  
|                    | Viale delle Scienze (Parco d'Orleans)  
|                    | 90128 Palermo  
|                    | Tel: +09123863710  
|                    | Fax: +39091702520  
|                    | Email: clelia.dispenza@unipa.it |
| 10. Japan          | Mr. Yasunari Maekawa  
|                    | Japan Atomic Energy Agency (JAEA); Quantum Beam Science Directorate; High Performance Polymer Group  
|                    | 1233 Watanuki-Machi  
|                    | Takasaki, Gunma-ken 370-1292  
|                    | Tel: +81 27 346-9410 |
| 11. Korea  | Mr. Kwang-Pill Lee  
Department of Chemistry Education, Teacher's College  
Kyungpook National University  
1370, Sankyuk-dong, Buk-gu, Daegu 702-701, South Korea  
Tel:+82-53 950-5901  
HP:010-3006-5901  
Fax:+82-53 952-8104  
E-mail: kplee@knu.ac.kr  
Homepage: www.kplee.com |
| --- | --- |
| 12. Malaysia  | Mr. Mohd Yusof Hamzah  
Makmal Nanoteknologi  
Blok 64, BTS  
Jafan Denghil  
Agensi Nuklear Malaysia  
Bangi  
43000 Kajang, Selangor  
Tel: +60389250510 ext:1493  
Fax: +60389282963  
E-mail: m_yusof@nuclearmalaysia.gov.my |
| 13. Poland  | Mr. Slawomir Kadłubowski  
Technical University of Lodz  
Wroblewskiego 15  
93-590 Lodz  
Phone: + 48426313161 (office)  
Fax: +48 42 6840043  
Email: slawekka@mitr.p.lodz.pl |
| 14. Serbia  | Ms. Aleksandra Krkljes  
Vinca Institute of Nuclear Sciences  
Laboratory for Radiation Chemistry and Physics  
"GAMMA" (030)  
P.O. Box 522  
11001 Belgrade  
Phone: +381 (0)11 8066428  
Fax: +381 (0)11 3408607  
E-mail: krkljes@vinca.rs |
| 15. Thailand  | Ms. Wanvimol Pasanphan  
Kasetsart University; Faculty of Science; Department of  
Applied Radiation and Isotopes  
50 Phahonyothin Road, Chatuchak  
Bangkok 10900 |
| 16. Turkey | Mr. Olgun Guven  
Hacettepe University  
Department of Chemistry  
06800 Ankara  
Tel: +90-312-297-7977  
Fax: +90-312-297-7973  
Email: guven@hacettepe.edu.tr |
| 17. USA | Mr. Allan S. Hoffman, ScD  
Bioengineering Department  
Box 355061—Foege, room N530R  
1705 NE Pacific St.  
University of Washington  
Seattle WA 98195-5061  
Phone: 206-543-9423  
Email: hoffman@u.washington.edu |
| **Cost Free Observers** |
| 18. Brazil | Mr. Gustavo Henrique Costa Varca  
Rua Henrique Lamberti n 292,  
Jardim Emília, Sorocaba,  
Sao Paulo 18031-020  
Home: (55-15) 32336330  
Mobile: (55-15) 8114-9886  
Email: ghvarca@ipen.br |
| 19. France | Ms. Marie Clochard  
Ms. Marie-Claude Clochard  
CEA  
Saclay  
91191 GIF-SUR-YVETTE  
Cedex  
France  
Telephone: +33 (0) 1 69 33 45 26  
Facsimile: +33 (0) 1 69 33 45 54  
Email: clochard@cea.fr ;  
marie claude.clochard@polytechnique.edu |
<table>
<thead>
<tr>
<th><strong>Scientific Secretary</strong></th>
</tr>
</thead>
</table>
| **20. IAEA** | Ms. Agnes Safrany  
|               | NAPC/IACS Industrial Applications and Chemistry Section  
|               | Division of Physical and Chemical Sciences  
|               | International Atomic Energy Agency  
|               | Wagramerstrasse 5  
|               | P.O. Box 100  
|               | A-1400 Vienna  
|               | Austria  
|               | Telephone: (+43 1) 2600-21750  
|               | Fax: (+43 1) 26007-21750  
|               | Email: a.safrany@iaea.org |