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Collagen - Modified Layered Silicate Biomaterials for Regenerative Medicine of Bone Tissue

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1. Introduction

The interdisciplinary field of polymer nanocomposite biomaterials brings together researchers from polymer science, biology, materials and biomedical engineering, chemistry and physics. In order to understand the need of biomaterials in our life, first we need to define and understand the specific terms that link these fields together. It is obvious that, due to the fact that the field of biomaterials is quite a new interdisciplinary one, the same term can be found with several explanations.

“The application of the principles of natural sciences, especially of biology, biochemistry and physiology to clinical medicine” was defined as **biomedicine** (Miller & O’Toole, 2005, as cited in Medical Dictionary, ndc; Picket, 2000, as cited in Medical Dictionary, ndc).

Collaboration between fields leads to the generation and use of new terminology and definitions that are used across each discipline in part. For example, the traditional definition of biomaterials has changed in time (Wu et al., 2010) as these materials find their use in a variety of medical and nonmedical technologies that are inspired by biology (Huebsch & Mooney, 2009; Williams, 2009; Wu et al., 2010). In 1987, The European Society of Biomaterials (Williams, 1987) defines the biomaterial as “a non viable material used in a medical device, intended to interact with biological systems.” Later on, in 1999 (Williams, 1999), in the dictionary of biomaterial science, the definition of biomaterial was changed, saying that it represents “a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body” (Williams,

2009). In a 2003 medical encyclopedia (Miller & O'Toole, 2005 as cited in Medical Dictionary, ndb) the authors combine the 1999 and 2002 definitions saying that the biomaterial can be defined as "any substance (other than a drug), synthetic or natural, that can be used as a system or part of a system that treats, augments, or replaces any tissue, organ, or function of the body; especially, material suitable for use in prostheses that will be in contact with living tissue". In 2005, modern medicine (Segen, 2005 as cited in Medical Dictionary, ndb) gives two definitions for biomaterial as being - "1. any synthetic material or device - e.g. implant or prosthesis - intended to treat, enhance or replace an aging or malfunctioning-or cosmetically unacceptable - native tissue, organ or function in the body, bioengineering, breast implants, hybrid artificial pancreas, Shiley valve, teflon, total hip replacement and 2. a biomaterial used for its structural, not biological properties - e.g., collagen in cosmetics, carbohydrates modified by biotechnology to be used as lubricants for biomedical applications or as bulking agents in food industry." The latest definition in 2009 (Williams, 2009), which includes all the previous ideas, states that "A biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine." Facing this changes in time, makes it difficult to classify polymer nanocomposite biomaterials into materials which are intended for medical devices and which implies their use for bio-technological purposes (e.g., renewable resources) (Wu et al., 2010). Polymeric biomaterials are developed in different shapes and for different purposes in order to substitute and repair biological tissues (Hench & Polak, 2002; Ma & Elisseff, 2005, as cited in Wu et al., 2010). New attempts to design self-assembled and smart nanocomposite biomaterials are conducted to fulfil some external requirements such as optic, temperature, mechanic, electric and magnetic fields (Hirst et al., 2008; Peppas et al., 2006; Stuart et al. 2010). These requirements are very important when designing smart implants and drug delivery systems as well as new bio-technologies including biosensors, *in vitro* diagnostics, cell culture matrices, contrast agents and bioassays (Hirst et al., 2008; Stuart et al. 2010; Wu et al., 2010).

Initially the implants were intended mainly to replace affected (i.e., broken bones, heart, vein, liver, pancreas) or malfunctioning body parts which people cannot live without. Subsequently, the industry of implants developed fast and now there are implants which are used for reconstructing and aesthetic purposes such as breast implants, bone facial implants and skin implants. According to the type of replaced tissue, they are divided into hard and soft implants. Hard tissue implants are usually made of metals or ceramics (bone implants, dental sealant, and joints) while soft tissue implants are made of polymers (blood vessels, skin, plastic surgery).

Essential components of modern medicine have become orthopaedic implants (represented by metallic implants as well as bone cements) (Widmer, 2001).

In recent years, orthopaedic implants and prostheses have imposed an increasing demand on the materials used. The more complex the function of the implant becomes, the more complex the requirements of the constructional material are. The success or failure of any device is often dependent on the choice of material as it is on the configurational and functional design.

The requirements that modern bone implants are expected to meet can be divided into three groups:

1. The biocompatibility between the material and the surrounding environment.
2. The mechanical and physical properties necessary to achieve the desired function.
3. The relative ease of fabrication and supply of the required components (Hoepfner & Chandrasekaran, 1994).

These requirements derive from the bone complex structure and properties. Bone is a complex and dynamic living tissue, which from morphological point of view can be divided into two types: cortical or compact bone and trabecular or cancellous or spongy bone. The mass of the skeleton is composed of 80% compact bones and 20% spongy bones. Cortical and trabecular bones contain the same cells and extracellular matrix (ECM) components, except that they are organized in a different way. The trabecular bone consists of a porous matrix with interconnected columns filled with bone marrow and is being responsible for metabolic functions of the bone, while the cortical bone contains fewer spaces, forms the external layer of all bones and provides them protection and load-bearing capabilities (Baron, 2003, as cited in Wilson, 2011).

Extracellular matrix is the most abundant component in bone tissue and is composed from 30% of an organic phase of collagen and of other proteins and from 70% of an inorganic phase of calcium phosphate. The organic phase provides elasticity and strength, while the inorganic phase gives hardness and load-bearing capabilities (Mundy et al. 2003, as cited in Wilson, 2011; Robey & Boskey, 2008, as cited in Wilson, 2011).

Remodelling is a physiological process in which the bones are being built, resorbed and then rebuilt again and that process is carried out and carefully controlled by a variety of cell types. Osteoblasts synthesize and mineralize the bone matrix which is maintained by osteocytes, and as required, is resorbed by osteoclasts (Wilson, 2011).

A biomaterial to be used as scaffold in tissue engineering must satisfy a number of requirements. These requirements include biocompatibility, biodegradation to non-toxic products within the period required for the application, processability to complicated shapes with appropriate porosity, ability to support cell growth and proliferation, ability to incorporate cells, growth factors, appropriate mechanical properties, as well as maintaining mechanical strength during the tissue regeneration process. All these requirements need to be fulfilled by implanted materials in order to achieve "biointegration". However, the term of biointegration is not completely accepted by researchers in the medical field (Szmukler-Moncler & Dubruille, 1990). Dubruille et al. did not agree to define biointegration as "describing the direct biochemical bonding of bone to an implant surface". For this situation they propose a new term "osseointegration" since the "biointegration" does not refer only to the integration with bone. Even though, we will find that biointegration term is still used and universally accepted as referring to the bioactivity of implanted material with bone tissue (Blokhuis et al., 2000).

Biocompatibility was defined as being "the property of being biologically compatible by not producing a toxic, injurious, or immunological response in living tissue" (Pickett, 2000, as cited in Medical Dictionary, nda).

In biology, the term "cell growth" is used in two different ways. In the context of reproduction of living cells the phrase "cell growth" is used for the idea of cell reproduction meaning growth in cell numbers. During cell reproduction the "parental" cell divides into two "daughter" cells. In other contexts, "cell growth" refers to increases in cell size (Conlon &

Raff, 1999). The first definition of the cell growth is also attributed to the word “proliferation” which is defined as “to grow or multiply by rapidly producing new tissue, parts, cells, or offspring” (Picket, 2000, as cited in Medical Dictionary, n.d.) when we refer only to cells. So, in material science in order to differentiate between the two concepts, we refer to cell growth when the size of the cell increases and use the term proliferation when the number of the cells increases.

Another important issue that needs to be considered, when obtaining an implant, is body response. Upon implantation, cell adhesion and fibrous tissue encapsulation takes place as a defensive response of the body (Recum & Strauss, 1998, as cited in Shin & Schoenfisch, 2006; Wilson & Gifford, 2005). The wound healing response initiates as an immune response. There were differentiated four distinct stages of the wound healing which are met starting after few seconds until complete healing: hemostasis, inflammation, repair, and encapsulation. Bacterial infection that can occur due to bacterial adhesion is a serious problem that affects implanted materials during healing or in the case of extended periods of time (Dankert et al., 1986, as cited in Shin & Schoenfisch, 2006; Sawan & Manivannan, 2000, as cited in Shin & Schoenfisch, 2006). In order to overcome this problem, strategies are being developed based on control release of an anticoagulant or antimicrobial agent from implanted material (Christiansen et al., 2011; Hendricks et al., 2000; Shin & Schoenfisch, 2006; Williams, 2009).

Drug delivery systems (DDS) are new types of drug carriers, designed to improve the pharmacological and therapeutic properties of administered drugs (Allen & Cullis, 2004). Such systems are designed according to the type of administration. There are transdermal drug delivery systems (TDDS) used for external administration of drugs and liposomal and targeted drug delivery systems (LTDDS) for internal administration. TDDS present the advantages of avoiding hepatic first pass metabolism, of decreasing gastrointestinal effect and improving compliance, while LTDDS are used to reduce the dose and increase the activity of the drug at the precise site (Kshirsagar, 2000). DDS can be classified after their release mechanism into (Lager & Peppas, 1981):

1. Diffusion Controlled Systems
 - a. Reservoirs (membrane devices)
 - b. Matrices (monolithic devices)
2. Chemically Controlled Systems
 - c. Bioerodible systems
 - d. Pendant Chain systems
3. Swelling Controlled Systems
4. Magnetically Controlled Systems

The dose released can differ according to the type of the system used and can be: extended release, slow release and sustained release (Kshirsagar, 2000).

Generally, DDS present zero-order kinetics and they should be inert and biocompatible with the environment. As compared to the conventional way of drug administration, DDS have the advantages of being more comfortable for the user, easily administered, possess a high drug concentration, have good mechanical strength, safe and free from leaks. They also have biocompatible, non-toxic, nonmutagenic, non-carcinogenic, non-teratogenic and nonimmunogenic properties (Lager & Peppas, 1981).

One of the mostly researched biomaterials used in various fields of medicine is natural polymer-collagen. Collagen is the most abundant protein in the body (skin, bones, teeth, tendons, cartilage, basement membrane, cornea, etc.). It can be used by different processing techniques in large various molecular structures (micro and nanostructures) as powder, injectable solutions, films, membrane and matrices (sponges).

The use of collagen as biomaterial, biocompatible and bioresorbable for the connective tissues prosthesis in which collagen is the basic protein is very well known.

To use collagen as scaffold in the recovery of bone tissue modifications in the structure and composition of matrix we need to obtain osteoinductive and osteoconductive effect. This can be achieved by the formation of biocomposites with SiO_2 , TiO_2 , clay, hydroxyapatite, etc (Christiansen et al., 2011; Vuluga et al., 2008a). Osteoinduction is the effect that appears in bone healing and involves the recruitment and stimulation of immature cells to develop into preosteoblast cells. Osteoconduction refers to bone growth on an implanted material, including bone conduction on its surface or down into pores, channels or pipes (Albrektsson & Johansson, 2001). Osteoconductive effect depends not only on biological factors, but also on the response to a foreign material. Bone conduction is not possible on certain materials, like some metals (Cu, Ag) (Albrektsson, 1995, as cited in Albrektsson & Johansson, 2001), while osteoinductive effect was observed on a large number of biomaterials (Barradas et al., 2011).

The layered silicate improves the thermal and mechanical properties of collagen and has a biostimulative effect on cellular metabolism. The structure of natural silicate would be like the structure of the living tissue because of the ionic exchange properties in aqueous medium just like the physiological phenomena in the human body where permanent information exchanges are present. Silicates surface can be modified with different bioactive substances and obtained nanohybrids can be dispersed in a collagen matrix (Potarniche et al., 2010; Potarniche et al. 2011). Depending on the concentration and the method of the silicate incorporation in the collagen protein, nanostructured systems with different morphologies, capable to control release of active principles (antibiotics, anti-inflammatory, enzymes, etc.) can be obtained. These types of nanocomposites can be used in regenerative medicine of bone tissue (Christiansen et al., 2011).

Polymer/inorganic nanocomposites are materials with unique properties which have found applications in large fields of activity: aircraft industry, automobile, packaging, construction, electronics, electrical engineering and last but not least in medicine and pharmaceutical industry. To obtain nanocomposites hybrids, the clay minerals (hydrated layered silicates) are preferred due to their high mechanical and chemical strength and low cost. The biomedical properties of clays are known since antiquity, these being in principal antiseptic, bactericides, scar action, antitoxic, without microbial germs. The special surface physical and chemical properties, such as high specific surface, layer charge, swelling capacity, make clay and clay minerals to be useful for human health in both external and "per os" (internal) applications. Clays can be used as active principles or excipients in pharmaceutical formulas, cosmetic medicine, in treating intestinal disorders and as drugs, being good adsorbents and mucostabilizers (Choy et al., 2007).

In medicine, synthetic polymers are also frequently used, not because of their biological activity, but because they are relatively easy to obtain, they are inert to body fluids, they are

cheap and available. From the synthetic polymers used in biomedicine, the copolymers of maleic anhydride (MA) were selected, because of their "drug-delivery" properties. In the field of medical and pharmaceutical applications, MA copolymers are used as components of some polymer - drug systems with controlled release (Gupta et al., 2002). Using MA copolymers for pharmaceutical and biomedical applications has advantages related to the structure, activity and their properties. MA copolymers have, in general, a relatively well characterized and reproducible alternating structure, hydrophilic or hydrophobic character and charge density can be varied by appropriate choice of comonomer (comonomers) (Edlund & Albertsson, 2002). Although polyanhydrides are best to the drug delivery application, they were not used in orthopedic implantation due to low load bearing and mechanical properties. However, MA copolymers with considerable enhanced mechanical properties were specially designed for the orthopedic applications such as poly (anhydride-e-imides) which can be used as scaffold for bone tissue engineering application (Sabir et al., 2009).

In this chapter, we intend to present the obtaining and properties of new types of nanocomposites based on natural polymer (collagen) and bioactive compounds (natural layered silicate modified with maleic copolymers) which can be used as biomaterial, scaffold for bone tissue regeneration.

Binary nanocomposites of layered silicate/maleic copolymers can contain different bioactive substances which can be released in time and at low and controlled concentrations (bone growth factors, titania or carbon aerogels, hydrolyzed collagen, anticancer compounds, antimicrobial antioxidants, etc.).

Obtaining of collagen/modified layered silicate nanocomposites implies:

- layered silicate purification for usage in the biomedical field;
- modification of the layered silicate surface (organophilization) with maleic copolymers thus obtaining binary nanocomposites;
- uniform dispersion of binary nanocomposites in collagen gel thus obtaining ternary nanocomposites from which, by lyophilization, spongy matrices can be obtained.

The binary nanocomposites have high thermal stability which can allow the usage of some bioactive substances activated by local heating. These nanocomposites are compatible with collagen gel in such away biocompatible ternary nanocomposites matrices useful for regenerative medicine of bone tissue can be obtained.

2. Materials and characterization methods

2.1 Materials

2.1.1 Collagen gel

Collagen gel (CG) is a polydisperse colloidal system which consists in molecules with native triple helix conformation and it is extensively used to obtain collagen-based biomaterials. Collagen gel is defined as a system with intermediate properties which vary between a liquid and a solid. It is characterized by structural viscosity, pseudoplastic behaviour and its average molecular weight is about 300000 Da, a value corresponding to the native collagen molecule weight (Trandafir et al., 2007).

Mild chemical and/or enzymatic treatments allow the extraction of collagen from calf hide into aqueous medium with preservation of triple helical structure of molecules, of microfibrils and fibrils. The collagen obtained is a gel, type I fibrillar collagen.

Type I collagen gel at a concentration of 2.3% and pH = 3 is obtained from calf hide by the current technology used in the Collagen Department in Leather and Footwear Research Institute, Bucharest, Romania (Albu, 2011; Budrugaac et al., 2003; Siapi et al., 2005). The obtained gel is physically-chemically characterized by 2.17% protein substance and 0.13% ash contents. The circular dichroism results show the technology used allows obtaining type I collagen with a high degree of purity and with triple helical structure (Albu, 2011). Absence of fat from this gel shows the high degree of purity. We use 1.2% gels to obtain collagen biomaterials.

2.1.2 Purified bentonite (natural layered silicate)

Purified bentonite (natural layered silicate) characterized by 82% montmorillonite (MMT), by basal spacing, $d_{001} = 14.7 \text{ \AA}$, by 8% weight loss in the range of temperature $20 \div 200 \text{ }^\circ\text{C}$ (TGA) and by $R_{1,0}=0\%$; $R_{0,5}=0,2\%$; $R_{0,063}=65,8\%$; $R_{0,04}=97,1\%$, $R_{0,025}=100\%$, residue on sieve is obtained by purification of Romanian sodium bentonite.

2.1.3 Maleic copolymers

Maleic copolymers having variable hydrophobicity (maleic anhydride copolymers with a hydrophilic comonomer - vinyl acetate and a hydrophobic comonomer - methyl methacrylate), are obtained in anhydrous form by "Petru Poni" Institute of Macromolecular Chemistry, Romanian Academy, Iasi.

The monomers, catalysts, and solvents are carefully purified before usage, according to already known methods (Caze & Loucheux, 1975 as cited in Chitanu et al., 2006; Riddick & Bunger, 1970 as cited in Chitanu et al., 2006).

The MA-VA copolymers of maleic anhydride (MA) with vinyl acetate (VA), (Chitanu et al., 2006) and MA-MMA copolymers with methyl methacrylate (MMA), (Chitanu et al., 2007) are synthesized by free radical polymerization, purified by extraction and washed with diethyl ether. The composition is estimated by conductometric titration with aqueous 0.1N NaOH in 1:1 acetone-water mixture and the molecular weight (M_w) by viscosimetric measurements in acetone at $30 \text{ }^\circ\text{C}$.

The chemical structures of maleic copolymers used are presented in Fig. 1 and 2.

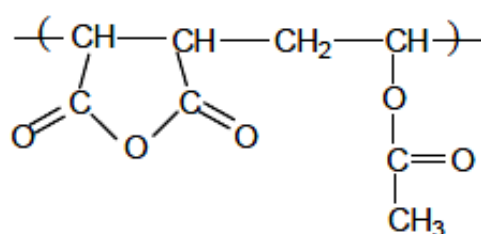


Fig. 1. Chemical structure of MA-VA copolymer.

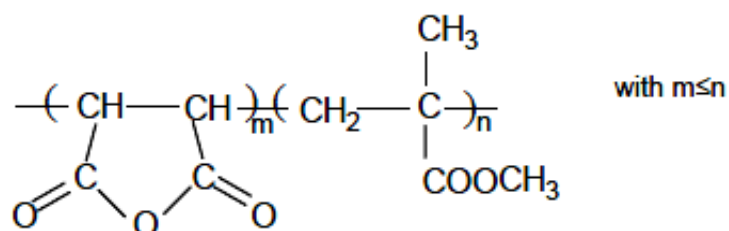


Fig. 2. Chemical structure of MA-MMA copolymer.

The characteristics of MA-MMA maleic copolymers with hydrophobic comonomer and of MA-VA maleic copolymers with hydrophilic comonomer are presented in Table 1.

Characteristics	MA-VA	MA-MMA
Average viscosimetric molecular weight, M_w	96000 g/mole	49000 g/mole
Acid index	0.481 g NaOH/ g copolymer	0.2535 g NaOH/ g copolymer
Composition	maleic anhydride : vinyl acetate 1 : 0.77	maleic anhydride : methyl methacrylate 1 : 2.17

Table 1. The characteristics of maleic copolymers.

2.2 Methods of physical-chemical and structural characterization

Physical-chemical and structural properties of purified layered silicate, of binary nanocomposites based on layered silicate/maleic copolymers and of ternary nanocomposites of collagen/layered silicate/maleic copolymers are determined by using different techniques described below.

2.2.1 X-ray diffraction

X-ray diffraction technique is used to determine the basal spacing. Diffractograms are recorded on automatic system using a DRON-20 diffractometer, with a horizontal goniometer and a scintillation counter. We used as radiation source, CoK_α ($\lambda = 1.7902 \text{ \AA}$) filtered with Fe in order to remove k_β component from the Bragg-Brentano system (in reflexion mode). Basal spacing is determined using Bragg equation¹:

$$n\lambda = 2d \sin \theta, d = \frac{n\lambda}{2 \sin \theta} \quad (1)$$

where n represents the reflexion order, λ is the wavelength of X-rays, θ is the diffraction angle, d is the distance between the planes of crystalline network which produces the diffraction.

2.2.2 Scanning electron microscopy (SEM)

Nanocomposite particles shape and dimension are observed using a scanning electron microscope and microanalyzed with an energy dispersive X-ray spectrometer (EDAX). The

samples are studied at different magnitude orders using a HITACHI scanning electron microscope model S-2600N equipped with an energy dispersive X-ray spectrometer (EDAX).

2.2.3 Dynamic diffusion of light with laser source (DLS)

Using a Zeta Seizer MALVERN UK, the average diameter of submicron particles is determined in aqueous suspension ranging $3 \text{ nm} \pm 1 \mu\text{m}$.

2.2.4 Thermal analyses

Thermal behavior of the obtained materials is analyzed by determining the sample weight loss when heated at a constant rate (TGA), depending on temperature. By differential thermal analyses (DTA) we determine the temperature at which we get the highest decomposition rate. Thermal tests are performed on THERMAL ANALYST DUPONT 2100 using:

- heating rate: $20 \text{ }^\circ\text{C}/\text{min}$;
- in air with the flow rate of $50 \text{ cm}^3/\text{min}$;
- temperature range: $0 \div 700 \text{ }^\circ\text{C}$.

2.3 Biocompatibility

Biocompatibility properties are evaluated on already obtained binary/ternary systems using *in vitro* tests. Biocomposite matrices are cut into small samples (1 cm^2) and are sterilized in ultraviolet light on the both sides. The samples are sown with osteoblast cells from G 292 cellular line at an initially cellular density of 3.5×10^5 cells/plate. The obtained cultures are hatched at $37 \text{ }^\circ\text{C}$ in moist atmosphere with $5\% \text{ CO}_2$, then monitored from cytomorphologically point of view at 24 and 72 hours and from cellular viability point of view after 72 hours since hatching. After 24 hours, the matrices are observed under a Nikon TS 100 microscope in phase contrast and pictures are taken using a Nikon Coolpix 4500 digital camera. After 72 hours, the cultures are stained with hypericin and pictures are taken.

3. Bentonite purification

Knowing that the bentonite may contain different percentages ($5\div 40\%$) of quartz and other impurities, it is necessary to purify the natural layered silicate (bentonite). These impurities must be removed because in the surface modification process, in order to ensure the compatibility between the silicate and polymer matrix, they act as a sterile, hindering the process (Carrado, 2006).

Purification is made by dispersing the bentonite in distilled water at $60 \div 90 \text{ }^\circ\text{C}$. Quartz and other hydrophobic impurities are separated by decantation.

Small amounts of bentonite (approximately 5 g) are added into 100 ml of hot distilled water ($60\div 90 \text{ }^\circ\text{C}$) under mixing conditions. The mixing is followed in a ball mill for 2 hours until the suspension becomes homogenous. The obtained mixture is allowed to settle for 72 hours, then the upper layer is separated (the purified suspension) and dried for 24 hours at

45 °C. Purified bentonite results (PB), which is ground to obtain a powder with < 60 µm size particles.

Bentonite morphology, before and after purification, is highlighted through X-ray diffraction (XRD), thermo-gravimetric analyses (TGA) and scanning electron microscopy (SEM).

From X-ray diffractions we notice that small angle diffraction peaks present a higher surface area in purified bentonite as compared with the initial one, proving an increase of MMT concentration. We accept that MMT and cristobalite are the two main components in the bentonite analysed (without taking into account the impurity components). We calculate the concentration of MMT in initial bentonite based on the maximum diffraction intensities: 77% montmorillonite in initial bentonite and 82% montmorillonite in purified bentonite (Vuluga et al., 2008b).

The TGA results (Table 2) are in accordance with X-ray diffraction results. We notice that, after purification the mass loss on the second step of decomposition decreases, while the mass loss on the third step of decomposition increases proving the decrease of the impurities concentration and the increase of the amount of montmorillonite.

Sample	Weight loss on each decomposition step (%)			
	I 20÷200°C	II 200÷400°C	III 400÷550°C	IV 550÷700°C
Initial bentonite (B)	6.8	0.6	0.4	1
Purified bentonite (PB)	8.3	-	-	1.4

Table 2. TGA results for bentonite before and after purification.

From scanning electron microscopy images (Fig. 3 and 4) we notice the morphology of purified powder with submicron dimensions and with uniform arrangement of chemical elements as compared to the morphology of initial powder where chemical elements are relatively uniformly dispersed.

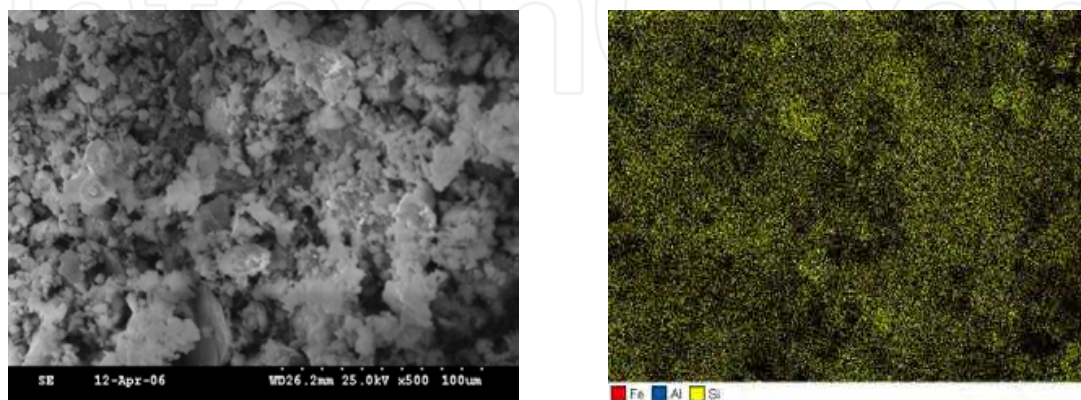


Fig. 3. Secondary electron images (SEI) and distribution of X-rays for initial bentonite.

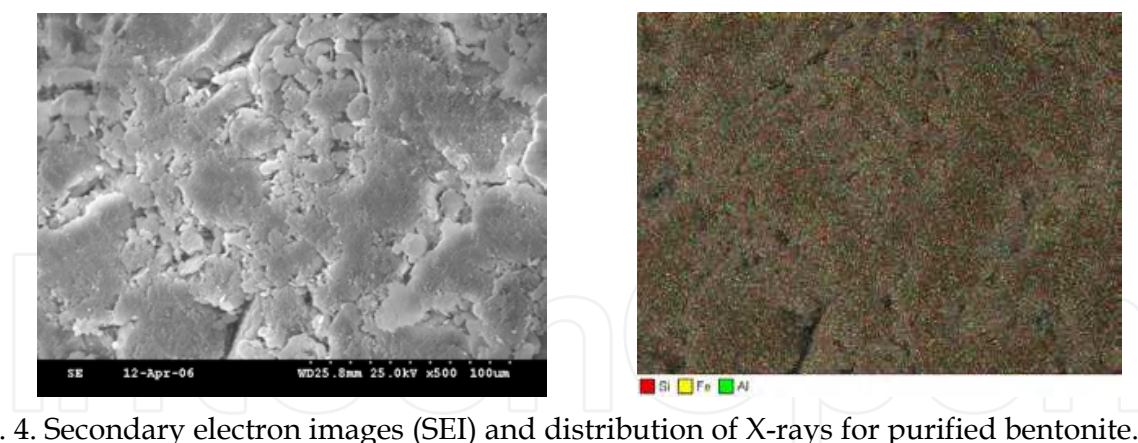


Fig. 4. Secondary electron images (SEI) and distribution of X-rays for purified bentonite.

4. Collagen/ modified layered silicate nanocomposites

Polymer/inorganic nanocomposites have attracted great recent attention and possessed a special position in constructing nano-assemblies due to their unique microstructure, outstanding properties and particular versatility. Nanocomposite materials can achieve much better properties than just the sum of their components as a result of interfacial interaction between the matrix and filler particles. It is the nature and degree of such interactions that play a pivotal role on the characteristics of resulted nanocomposites such as solubility, optical properties, electrical and mechanical aspects, biocompatibility, biodegradability. The organic-inorganic composites/hybrids represent a recent topic in which bibliographic references are still few. The year 2000 could be considered as the “starting point” in this field. Research for obtaining new types of bio-nanocomposites based on collagen in solid physical forms of matrices or membranes in which inorganic components can be hydroxyapatite, biovitroceramica, TiO_2 , SiO_2 , natural layered silicate as it is or modified with hydrolysed collagen, antioxidants has developed (Ficai et al. 2010a; Albu et al., 2009; Baia et al., 2008; Piticescu et al., 2008; Potarniche et al., 2010; Vuluga et al., 2007; Vuluga et al., 2008a). Organic compounds that can interact with collagen are represented by a series of biological active substances or other biocompatible polymers, their compatibility being previously tested with collagen gel or colloidal solutions. In such phases, the nanocomposites are obtained: membranes by free drying at 25 °C or matrices by freeze-drying process (lyophilization). According to the type of obtained nanocomposite, the amount of non collagen components should be: in case of matrices between 5÷20% insoluble powders and 30÷50% water soluble substances; in case of membranes between 1÷2% insoluble powders and 10-30% water soluble substances. Exceeding these amounts negatively influences the collagen matrix and membrane specific stability structure as well as the mechanical and hydrophilic properties. (Olteanu et al., 2008; Trandafir at al., 2007, Ficai et al., 2010b, Lungu et al., 2007, Titorencu et al., 2010).

Using hydrolyzed collagen modified layered silicates we obtain collagen based nanocomposites which can be used in healing varicose ulcer, the silicate improving the regenerative action of type I collagen on conjunctive tissue, reducing the healing time of ulcerous wound (Trandafir et al., 2007).

By introducing maleic copolymers as structural modifiers of layered silicates leads to binary stable hybrids compatible with collagen gel, a process which forms a liquid ternary nanocomposite resistant to further processing, for example to lyophilization.

4.1 Layered silicate/maleic copolymers binary nanocomposites

Until now, there has not been shown any interest in obtaining systems/composites/hybrids based on copolymers of anhydride or maleic acid with clays/bentonites. Literature is mostly made of patents which refer to synthesis or uses of systems in which the bentonite and the polymer are mixed without being associated with the composites or hybrids formation. One of the first areas, in which they are mentioned as systems of bentonite and maleic copolymers or polymers in general, is conducting drilling fluids for oil secondary recovery (Al-Marhoun et al., 1988; Gleason & Brase, 1985; Hale & Lawson, 1988; Hayes, 2005; Jarm et al., 1988; Libor et al., 1987; Martinko, 1987; Shaarpour, 2004). In medical field, different systems of bentonite/maleic polymers are obtained for dentistry uses (Heathman & Ravi, 2005), for parasites control (mites, moths) and water or dirt rejection (De Sloovere et al., 2004) and as dirt retention agents recommended for hand cleaning pastes (Florescu et al., 1994). In conclusion, we can say that the composite/organo-inorganic hybrids based on bentonite and maleic copolymers are a promising open field.

The natural layered silicate/maleic copolymer binary nanocomposites are obtained by solution intercalation method (Ray & Okamoto, 2003). According to the chemical structure of maleic partner, the interaction between inorganic and organic components is performed at different ratios (1: 1; 1: 2 and 2: 1), in a mixture of 1:1 ethanol- water. Thus, we obtain maleic copolymer modified layered silicate binary nanocomposites: PB/ MA-MMA (1: 1; 1: 2 and 2: 1) and PB/ MA-VA (1: 1; 1: 2 and 2: 1).

From particle size analysis of resulted binary nanocomposites (Fig. 5 and 6) we notice:

- the PB/ MA-MMA nanocomposite presents particle size dimensions at a range of 3000÷6000 nm;
- the PB/ MA-VA nanocomposite presents a similar behavior as that obtained with PB/ MA-MMA the difference being the particle size is bigger (at a range of 4000÷13000 nm);
- the higher the maleic copolymer concentration is the bigger the particle size; this is valid up to an approximately 50% concentration as compared to silicate particle size; an over 50% concentration is followed by a decrease in particle size up to a close value of unmodified silicate.

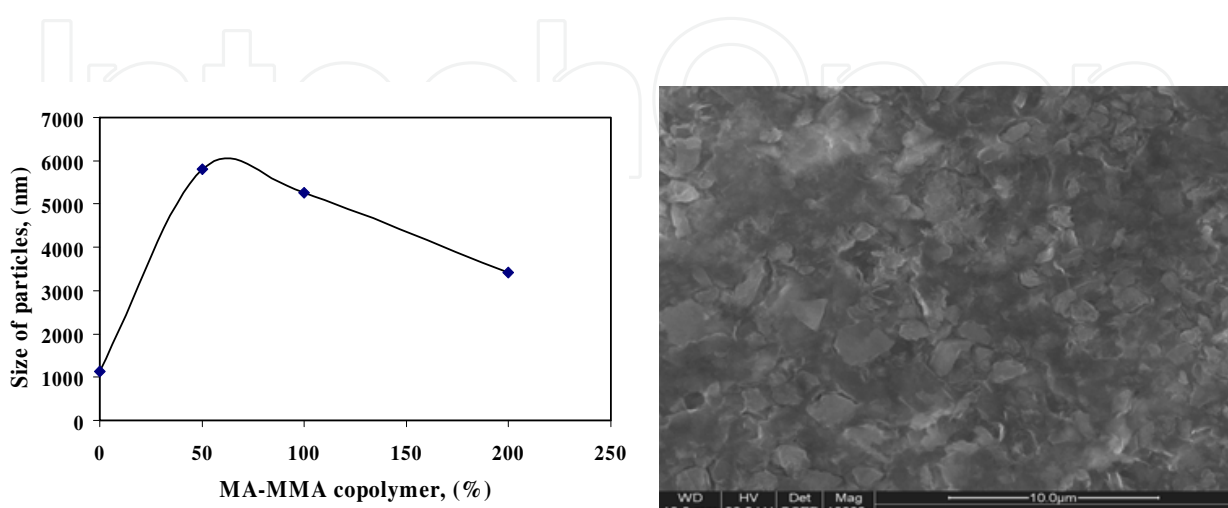


Fig. 5. Particle size and morphology for PB/ MA-MMA binary nanocomposites.

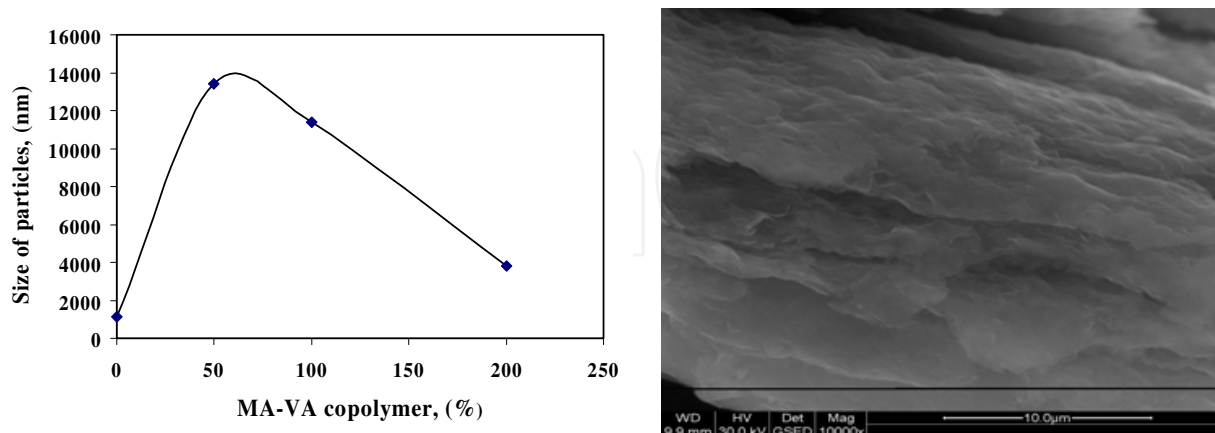


Fig. 6. Particle size and morphology for PB/ MA-VA binary nanocomposites.

From X-ray diffractions (Fig. 7 and 8) we notice that silicate structure changes in a specific way: a peak disappears at $2\theta = 23^\circ$, which is silicate characteristic peak and shapes very well the peak at approximately $2\theta = 3^\circ$. This proves the interaction between silicate and maleic copolymers as well as an organized structure. Intercalated nanocomposites are obtained with a lamellar ordered structure. Maleic copolymer with hydrophobic comonomer (MA-MMA) interacts better with silicate, the best structure is obtained at 1:1 silicate/copolymer ratio. In the case of maleic copolymer with hydrophilic comonomer (MA-VA), the best structure is obtained at a low amount of copolymer, meaning at 2:1 ratio. These two types of layered silicate/ maleic copolymer binary nanocomposites are selected to obtain ternary nanocomposites.

From thermal stability point of view, binary nanocomposites thermograms present differences according to hydrophilic or hydrophobic comonomer used to modify layered silicate.

As compared to purified bentonite, at which the maximum rate of decomposition is on the first step, at a temperature of 74°C , the organophilized bentonite presents a shift of maximum decomposition rate towards higher temperatures ($220\div 225^\circ\text{C}$ for PB/ MA-VA nanocomposites and $220\div 365^\circ\text{C}$ for PB/ MA-MMA nanocomposites), on the second step of decomposition, similar to maleic copolymers (Table 3). An increase of maleic copolymer concentration (from 50% to approximately 200% reported to bentonite) reflects into an increase of weight loss at the temperature range of $200\div 500^\circ\text{C}$. The obtained results show the intercalation of maleic copolymers between the silicate layers. Thermal stability of obtained binary nanocomposites correlates with the degree of interaction between silicate and copolymer. We notice that in the case of PB/ MA-MMA nanocomposites the best thermal stability is obtained at 1:1 ratio while the best thermal stability for the PB/ MA-VA nanocomposites is obtained at 2:1 ratio. These results are in accordance with X-ray diffraction results.

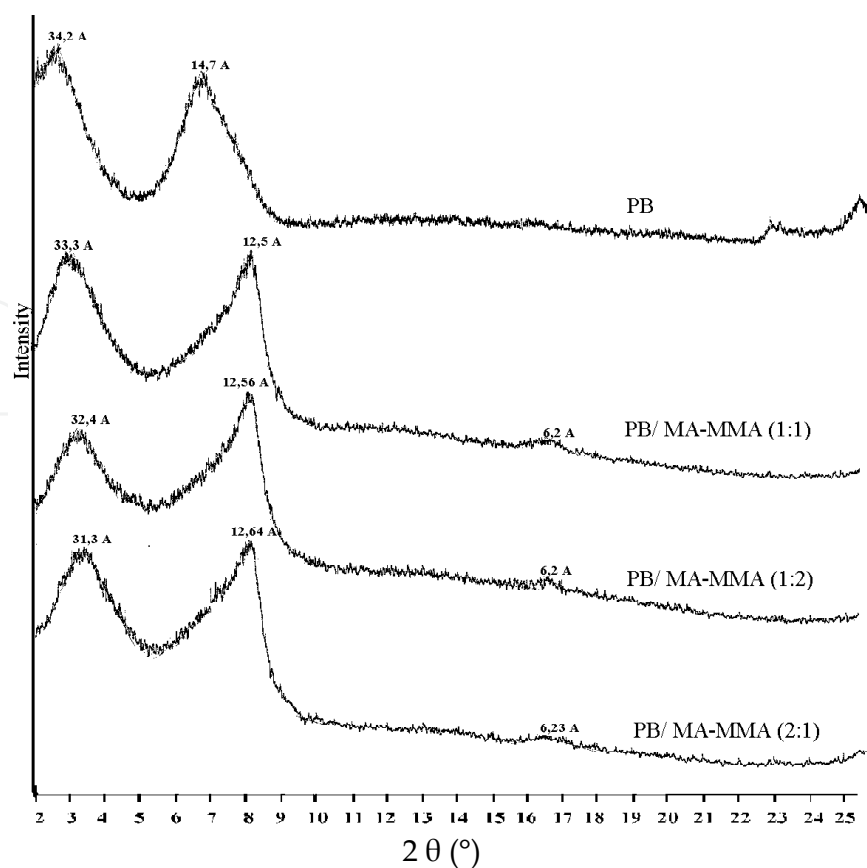


Fig. 7. X-ray diffractions for PB/ MA-MMA nanocomposites at different ratios.

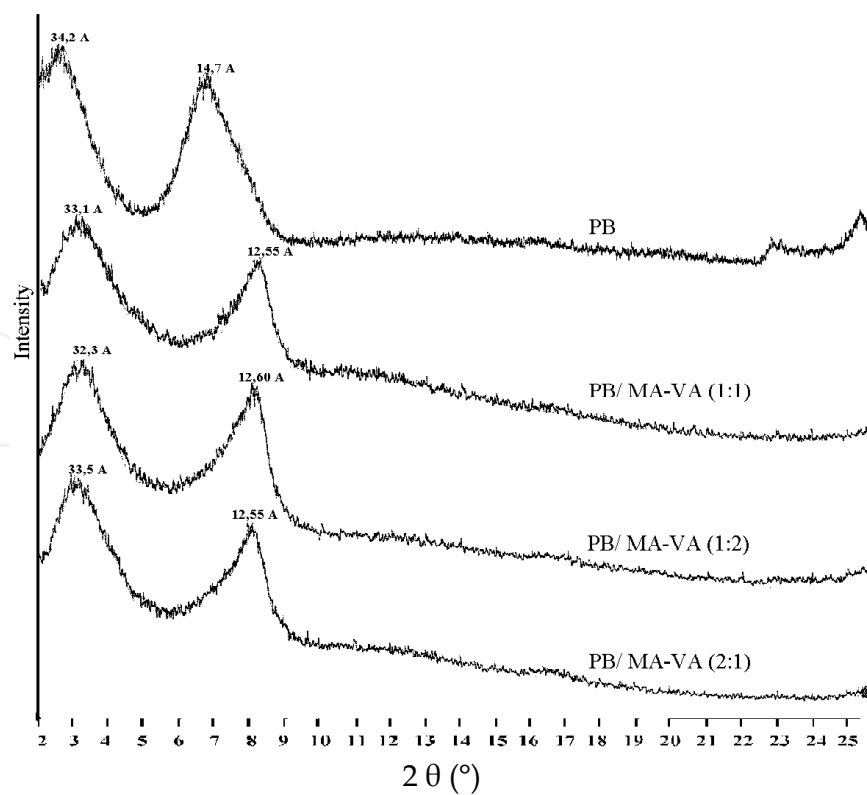


Fig. 8. X-ray diffractions for PB/ MA-VA nanocomposites at different ratios.

Sample	Weight loss on the each decomposition step (%) / T _{rdmax} (°C)			
	I 20 ÷ 200 °C	II 200 ÷ 400 °C	III 400 ÷ 550 °C	IV 550 ÷ 700 °C
PB	8.3/ 74	-	-	1.4
MA-MMA	4.9	84.3/ 321	8.1	2.7
PB/ MA-MMA (1:1)	10.7	38.5/ 367	6.7	1.6
PB/ MA-MMA (1:2)	10.4	52.1/ 365	8.7	1.3
PB/ MA-MMA (2:1)	8.4	22.1/ 218	7.5	5.4
MA-VA	7.6	61.3/ 258	27.9	0.9
PB/ MA-VA (1:1)	7.4	35.6/ 223	14.4	3.3
PB/ MA-VA (1:2)	7.1	27.2/ 220	8.2	6
PB/ MA-VA (2:1)	7.4	25.2/ 226	4.5	7.9

T_{rdmax} Temperature at the maximum rate on the first decomposition step

Table 3. TGA results for PB/ Maleic copolymer binary nanocomposites as compared to TGA results for maleic copolymers (MA-MMA and MA-VA).

4.2 Collagen/ layered silicate/ maleic copolymer nanocomposites

The layered silicate can improve thermal stability of collagen and depending on the collagen morphology (gel or colloidal solutions) and on the method used for silicate dispersion, a variety of lamellar structures and morphologies – intercalated or exfoliated- can be obtained (Vuluga et al., 2007).

It is well known that the interaction of collagen with maleic copolymers takes place prevalently through weak forces and, to a little extent, through electrostatic interaction between the carboxylic groups of copolymer and the aminic groups of collagen (Angelescu-Dogaru et al., 2004; Chitanu et al., 2002).

Maleic anhydride functionalized polymers ensure organosilicate dispersion and exfoliation into the polymer matrix (i.e. polypropylene) due to the interaction that may occur between maleic anhydride and hydroxyl groups on the silicate surface (Faisant et al., 1998; Vuluga et al., 2008b; Utracki, 2002).

Considering the results of these studies, it is reasonable to believe that a high adhesion to the collagen/modified layered silicate interface due to the interaction between the components leads to a uniform dispersion of modified layered silicate into collagen matrices and to improved properties.

A dispersion of 0.5% of layered silicate modified with maleic copolymer in water: alcohol (1:1) mixture is added, when stirring, at room temperature, into a solution of collagen with 1.2% dried substance to obtain ternary nanocomposites. The different types of nanocomposites obtained in liquid form (which contain 5-10% of MMT reported to collagen dried substance) are crosslinked with 0.1% formaldehyde, reported to collagen dried substance and are dried by lyophilization to obtain spongy matrices. The freeze-drying process is an advantageous conditioning technique which consists of drying frozen samples by ice sublimation into vacuum. To maintain the triple helix conformation of collagen molecules we use the following process: the liquid ternary nanocomposites are cast in stainless steel plates, cooling to -40 °C (4 hours), kept up for 4 hours, then freeze-dried at -

40°C and 0.12 mbar for 10 hours, subsequently heated to +20 °C at a rate of 3 °C/hour (20 hours) at 0.12 mbar, heated again (6 hours) to 30 °C at a rate of 2 °C/hour and finally freeze-dried at the same temperature at 0.01 mbar for 4 hours, using the Christ Model Delta 2–24 LSC freeze-dryer (Germany). During the 48 hours of freeze-drying we obtain: CG/ PB/MA-VA and CG/ PB/MA-MMA spongy matrices.

We study both the influence of binary nanocomposite type and of the collagen solution pH upon ternary nanocomposite properties. Although the normal human body pH is 7.2 we still study the nanocomposite properties at an acidic pH of 3 knowing that in acidic environment collagen molecules are positively charged by the amine groups thus being able to interact more easily with the negatively charged layered silicates.

Both ternary nanocomposites (CG/PB/MA-MMA and CG/PB/MA-VA) present a partially exfoliated lamellar structure (Fig. 9). In the nanocomposites with hydrophobic comonomer (MMA) the degree of interaction is stronger at basic pH, thus we obtain partially exfoliated nanostructured biomaterials. When we use hydrophilic comonomer (VA), the degree of interaction is stronger at acidic pH. For biocompatibility tests, we select the nanocomposite with hydrophobic comonomer. The strong degree of interaction for the hydrophobic comonomer reflects itself in an improved thermal stability (Table 4).

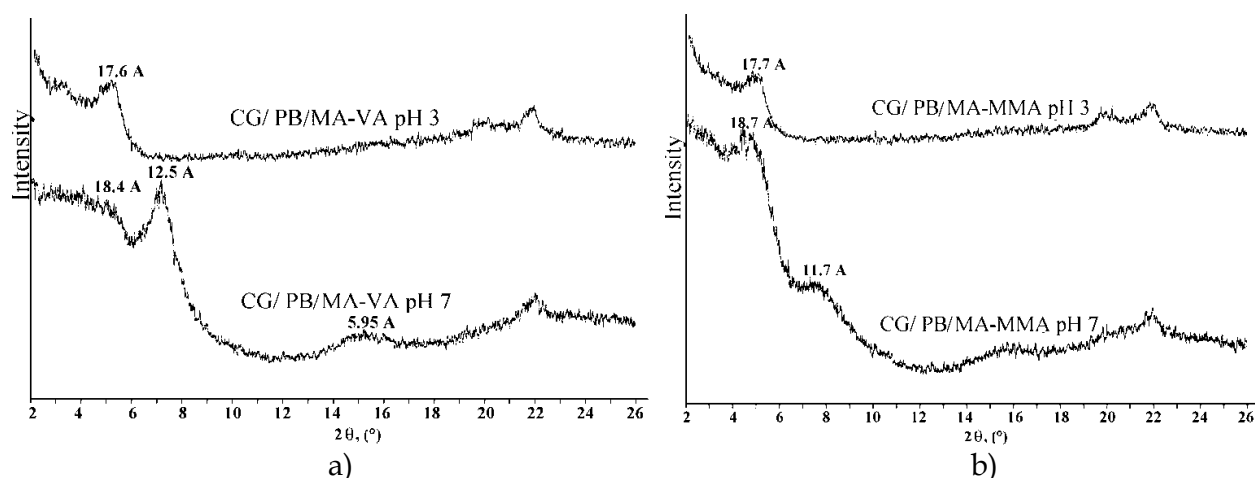


Fig. 9. X-ray diffraction patterns for ternary nanocomposites with hydrophilic (a) or hydrophobic (b) comonomer, at acidic and basic pH.

As compared to collagen, the CG/ PB/MA-MMA nanocomposites are more thermally stable. Thermal stability for the ternary nanocomposites is higher at basic pH and with hydrophobic maleic comonomer (Table 4).

The nanocomposites CG/PB/MA-MMA (pH 7) present a spongy structure with interconnected macro, micro and nano sized pores, with nanostructured regions (Fig. 10 a). Collagen morphologic structure presented in secondary electron images (SEI) shows regions with large communicating pores as well as interconnected ones. We also notice the morphologic homogeneity of the collagen fibers (Fig. 10 b). Even though the amount of maleic copolymer modified layered silicate in ternary nanocomposites is less than the amount of collagen, a compact fibrous structure can be observed, which can be the reason of an increase of thermal stability.

Sample	Residue at 700 °C, (%)	Weight loss on each decomposition step (%)/T _{vmax} (°C)		
		I	II	III
		0 ÷250 °C	250 ÷500 °C	500 ÷700 °C
Collagen Matrix pH 7.2	1.6	14.3/ 147	50.2/ 337	33.9/ 600
CG/ PB/ MA-VA nanocomposite matrix pH 3	16.3	13.1/ 171	40.8/ 344	29.8/ 588
CG/ PB/ MA-VA nanocomposite matrix pH 7	18.9	16.3/ 171; 207	39.3/ 345	25.2/ 605
CG/ PB/ MA-MMA nanocomposite matrix pH 3	15.4	11.6/ 173	48.9/ 349	24.1/ 585
CG/ PB/ MA-MMA nanocomposite matrix pH 7	23.4	9.1/ 87; 172	41.6/ 351	25.2/ 586

T_{vmax}, temperature at maximum rate of decomposition, (°C)

Table 4. Thermal stability for the ternary nanocomposite matrices.

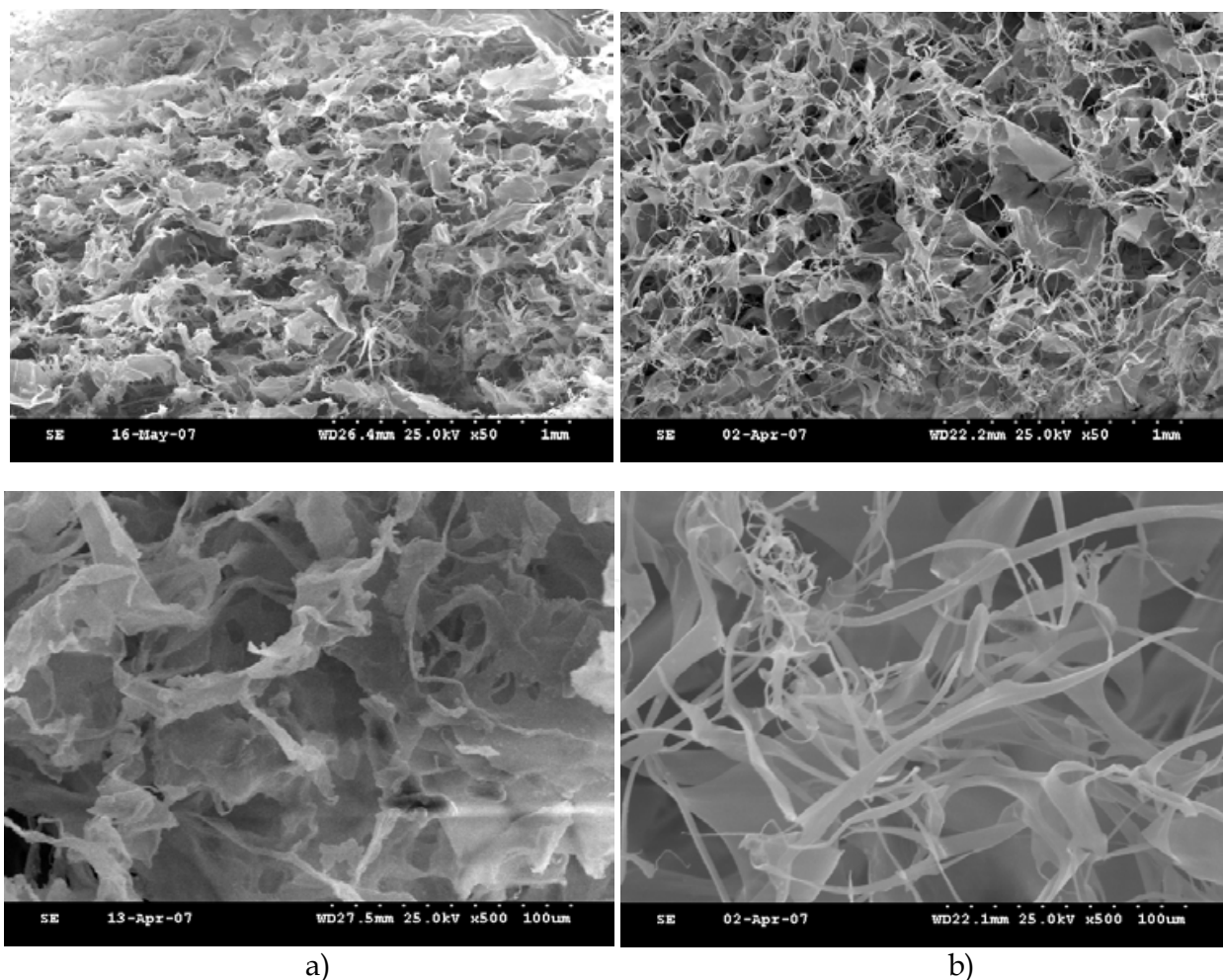


Fig. 10. Morphologic structure of (a) CG/ PB/ MA-MMA nanocomposites pH 7, compared to (b) collagen morphology.

The spongy matrix of CG/ PB/ MA-MMA nanocomposite was *in vitro* tested on osteoblast cell cultures. *In vitro* test results are presented in Table 5. After 24 and 72 hours since hatching, the cells proliferate on matrix surface, the viability being of 97%, very close to the reference value (control sample). These results are also supported by microscopy observation of cellular density (Fig. 11). The collagen/layered silicate/MA-MMA ternary nanocomposite does not present any cytotoxic response and the cells present normal phenotype.

Sample	Optical density DO 550nm	Viability from control sample, (%)
CONTROL	1.0263	100%
CG/ PB- MA-MMA nanocomposite matrix pH 7	0.9954	97%

Table 5. Proliferation and osteoblast cells viability hatched on matrix of CG/ PB/ MA-MMA nanocomposites.

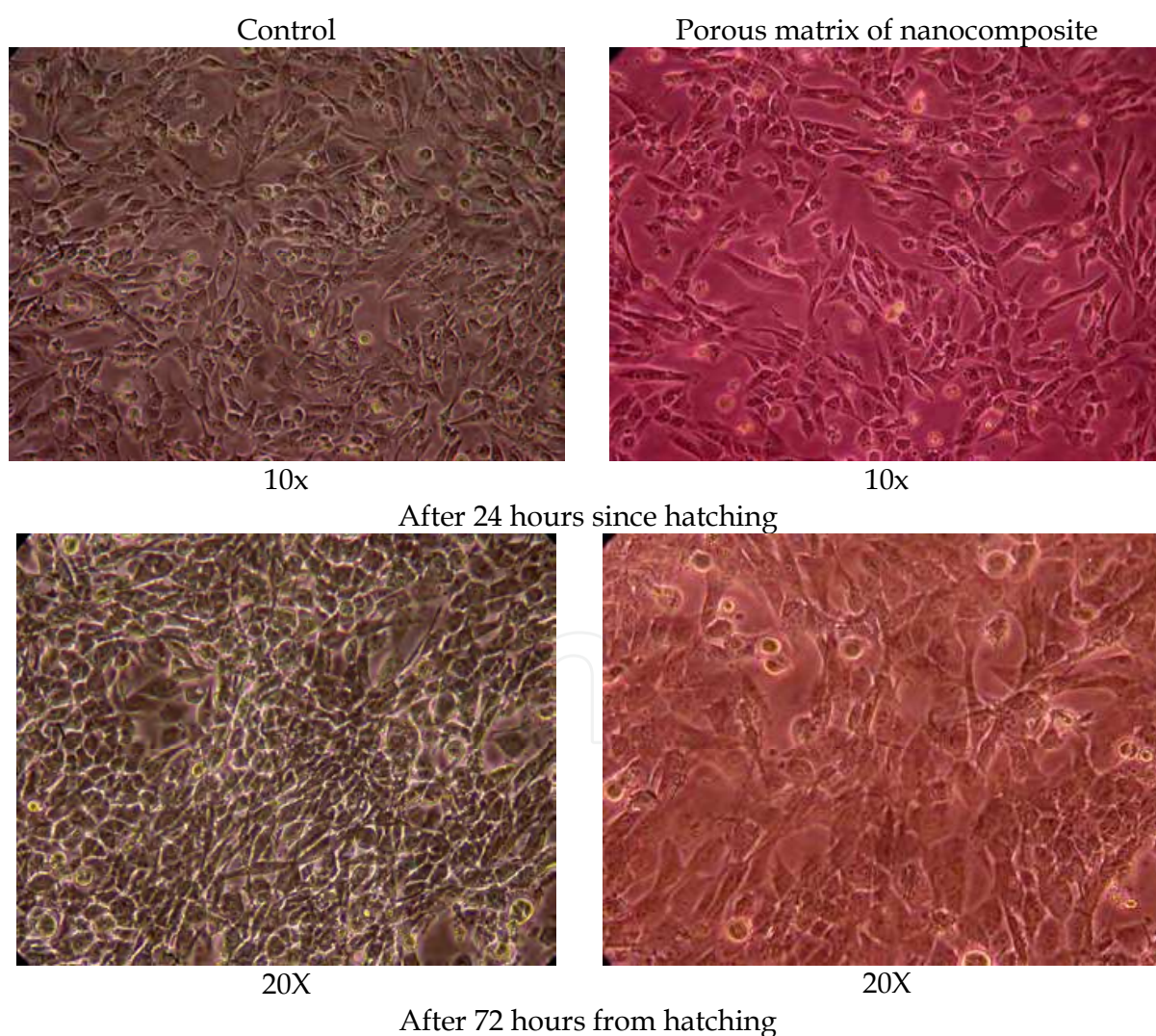


Fig. 11. Microscopic analysis of osteoblast culture samples sowed on CG/ PB/ MA-MMA ternary nanocomposite substrate after 24 and 72 hours since hatching.

5. Conclusions

The key results consist in obtaining new types of collagen nanostructured biomaterials in the form of spongy, microporous matrices, nontoxic and biocompatible with osteoblast cells. The preparation of the new nanocomposites is based on the use of two natural components: collagen, which contributes to bone regeneration and natural layered silicate (montmorillonite) which improves the thermal resistance of collagen, able to release in time an active substance at low and controlled concentrations. Maleic copolymers favour the silicate dispersion in the matrix of collagen and the obtaining of intercalated partially exfoliated nanocomposites with disordered lamellar structure. The nanocomposites in form of microporous matrix (scaffold) obtained by dispersion of binary nanocomposites in collagen gel have a spongy structure which contains macro and micro interconnected nanopores, similar with extra cellular matrix, which allows the penetration of the physiological fluids, necessary for the growth of cells. In comparison with collagen matrix, CG/ PB-MA-MMA nanocomposites have improved thermal stability.

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Materials are important to mankind because of the benefits that can be derived from the manipulation of their properties, for example electrical conductivity, dielectric constant, magnetization, optical transmittance, strength and toughness. Materials science is a broad field and can be considered to be an interdisciplinary area. Included within it are the studies of the structure and properties of any material, the creation of new types of materials, and the manipulation of a material's properties to suit the needs of a specific application. The contributors of the chapters in this book have various areas of expertise. therefore this book is interdisciplinary and is written for readers with backgrounds in physical science. The book consists of fourteen chapters that have been divided into four sections. Section one includes five chapters on advanced materials and processing. Section two includes two chapters on bio-materials which deal with the preparation and modification of new types of bio-materials. Section three consists of three chapters on nanomaterials, specifically the study of carbon nanotubes, nano-machining, and nanoparticles. Section four includes four chapters on optical materials.

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