SCUOLA DI ALTA FORMAZIONE

DOTTORATO IN BIOTECNOLOGIE PER L'UOMO

PHD PROGRAM IN BIOTECHNOLOGIES FOR HUMAN HEALTH

COORDINATORE: PROF. CLAUDIO SANTORO

Relazione annuale da compilarsi a cura del Dottorando

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Relazione scientifica dell'attività svolta (in inglese).

During the first year of my Ph.D. program, I worked in the Laboratory of Odontostomatology, in collaboration with the Laboratory of Histology, in the Department of Medical Science of University of Piemonte Orientale. I completed a pilot study about the possibility of adsorption of antibodies on biomimetic hydroxyapatite nanocrystals (HA-NC), with the future perspective of creating multifunctional complexes, using monoclonal antibodies as active targeting.

Absorption of human IgG on hydroxyapatite nanocrystals surface

Project Aims/Objectives

The overall goal of this project is to determine whether the adsorption of antibodies on HA-NC really occurs and to investigate the stability of the link. Therefore, we are addressing **the hypothesis that the surface of biomaterial can interact with peptides, generating an electrostatic bound**.
We will perform studies with the following specific aims:

1. **To synthesize and characterize HA-NC.**
2. **To ascertain the presence of antibodies on HA-NC.**
3. **To quantify antibodies loaded on.**
4. **To evaluate the stability of link between antibodies and HA-NC.**

Background

Nanomedicine

Cancer represents one of the principal cause of death for adults in developed countries. Because of its high complexity and heterogeneity, current diagnostic, prognostic and therapeutic tools are not enough to ensure a successful treatment (Hanahan and Weingberg, 2000; Liotta and Petricoin, 2000).
Nie et al. reported three of the major problems associated to contemporary oncology: *i)* to obtain advanced technology for tumour imaging and early detection, in order to develop a precise diagnosis and prognosis; *ii)* to produce anticancer drugs formulations able to discriminate between cancerous and normal cells, reducing toxicity and adverse effects of chemotherapy; *iii)* to improve basic knowledge in cancer biology (Nie et al, 2007).
Cancer nanotechnology, together with cancer biomarker advance, could lead to a "personalized oncology", where early tumour detection and diagnosis are more and more specific and sensible and therapy reaches its specific targets (Evans and Relling, 2004).

Originally referring to materials science, nanotechnology is related to the manufacturing of new and useful materials and devices with nanoscale dimension, between 1 and 100 nm (Liu et al, 2007; Sinha et al, 2006).
In general, nanometer-sized particles can be subdivided in two major groups, according to their chemical nature: organic (i.e. liposomes and dendrimers) and inorganic nanoparticles (NPs) (i.e. magnetic iron oxide, mesopourous silica particles and HA-NC). Each of them respects the fundamental rule of mimicking nature, to optimise their interface with biological tissues and, if necessary, to correctly *in situ* release bioactive molecules, following specific kinetic (Roveri at al. 2008).
In particular, NPs provide new and unique structural properties and represent one of the best device for nanomedicine application, such as the rational delivery and targeting of pharmaceutical, therapeutic and diagnostic agents in order to achieve a safer and more effective biomedical techniques (Liu et al, 2007).

Passive targeting

First of all, NPs have a great potential to enhance the accumulation of anticancer drugs in cancer cells rather than in healthy tissues. The limited pore size of tissutal endothelium is the primary delivery barrier for NPs, but it also suggests a selective accumulation in certain tissues, where endothelial walls show a defective architecture, as in cancer tissues. This phenomenon is called passive tissue selectivity (or "passive targeting"), based on the rapid vascularisation of fast growing tumour mass, resulting in leaky and malfunctioning vessels structure and in impaired lymphatic drainage which consent the so called "enhanced permeability and retention (EPR)" effect (Li and Huang, 2008).

RES and MPS clearance

Secondly, giving to NPs determined size and innovative surface properties, it is possible to improve their tissutal accumulation avoiding the clearance of the macrophages residing in the tissues, also known as reticuloendothelial system (RES) and mononuclear phagocytes system (MPS). In fact, when NPs are intravenously injected, a variety of serum proteins binds their surface, some of them (termed "opsonin") are recognized by the scavenger receptor on macrophage cell surface and internalized, leading to a significant loss of NPs from circulation. To minimize protein binding and reduce MPS uptake, several modification are proposed: i.e, polyethylene glycol conjugation to NPs surface, NPs synthesis with a mean diameter of approximately 100 nm and neutral NPs production (Li and Huang, 2008).

Active targeting

Finally, NPs show large specific surface area (SA= $4\pi r^2$) and several functional groups, providing high potential for molecules absorption and allowing the so called "active targeting". This approach is based on specific molecules interactions, such as lectin-carbohydrate, ligand-receptor and antibody-antigen (Nie et al, 2007; Allen, 2002). For instance, targeting ligand/antibody conjugated to the surface of NPs ("bioconiugate") can recognize and bind with the receptor (over)expressed on the target cell surface, triggering a receptor mediated endocytosis, which results in an increased level of intracellular delivery of the formulation (figure 1).

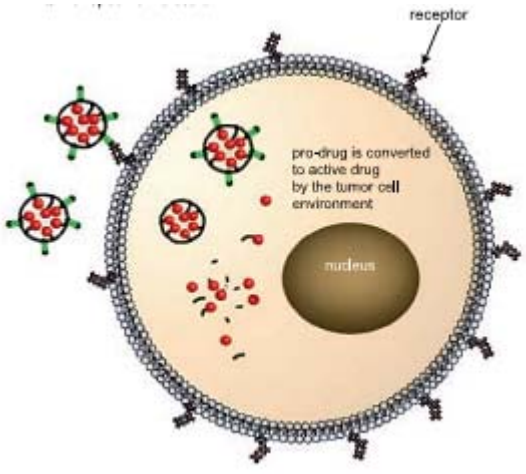


Fig. 1 "Stimulus responsive" drug delivery: NP contains the pharmaceutical agent (red point), while on its surface there is the targeting mediator (i.e ligand or monoclonal antibody). After ligand/antibody recognition, NP complex enters into the cell via lysosome/endosome pathway and in the cytoplasm the drug is released, by means of a stimuli responsive system.

Thus, ligand and antibodies used should have: high avidity and specifity for cell surface receptors (overexpressed in cancer cells), high rate of internalization, low immunogenicity, physiochemical properties, biodegradability and easily absorption onto NPs (Sinha et al, 2006).
It is noteworthy to underline as coating of a targeting ligand on NPs does not increase the level of accumulation of drug in the target tissue but increases the rate of intracellular delivery, being tissutal drug accumulation dependent from EPR effect (Li and Huang, 2008). Studies were conducted to create immunoliposomes and bioconiugates polimer-based NPs, till the development of "nanobodies", which utilize the smallest available intact antigen-binding fragments (Revets et al, 2005; Jain, 2006).
Despite the undoubted importance of the targeting-ligand approach, there are still some unsolved questions: *i)* there are few receptors that seems almost partially tumor-specific; *ii)* antibody-coniugated NPs exhibit greater blood clearance; *iii)* internalization by receptor mediated endocitosys is usually through the endosome/lysosome pathway, in which a great percentage of drug is trapped in the organelle and inactivated (Koning et al, 2002; Gabizon et al, 2004; Sinha, 2006; Li and Huang, 2008).

Triggered release

Afterwards to obtain pharmacological efficacy, the encapsulated drug must be released from NPs to the target cells. One of the major point of research is to correlate high levels of drug-NPs tumour accumulation with the bioavailability of the drug to tumoral cells, depending on the rate of drug release from NPs carrier delivery (Andresen et al, 2005; Li and Huang, 2008). To obtain a good local drug bioavailability, "triggered-release" mechanisms or "stimuli-responsive" systems became objects of study: i) to increase the rate of drug escape from endosome/lysosome by pH-dependent mechanism, using the acidic pH of endosome/lysosome; ii) to induct endosome rupture through osmotic pressure rise; iii) to utilize an internal/external physical triggering system (i.e. light triggered release by photo-excitation; heat-triggered release by hyperthermia; enzyme-triggered release by the employment of enzyme upregulated in the tumour tissue) (Li and Huang, 2008).

Disease specific NPs

The future perspectives of nanotherapeutics in cancer refer to the development of such "disease specific NPs" termed as "ternary NPs", because composed of three fundamental elements: i) a drug carrier; ii) a drug; iii) a ligand/antibody (Sinha et al, 2006; Jain et al., 2008; Liong et al, 2008).

HA-NC Functionalisation

In general, inorganic biomaterials appear to be more suitable than polymeric ones not only in replacing hard tissue, in the means of their mechanical behaviour, but also for injection drug delivery, having low toxicity and high hydrophilicity (Roveri et al. 2008).
Among them, synthetic hydroxyapatite nanocrystals (HA-NC) are one of the most interesting, now applied as bone substitute, in virtues of their excellent properties: biocompatibility, bioactivity, biodegradability, osteoconductivity and direct bone bonding. Bone and tooth exhibit, as major inorganic component, natural HA-NC with needle like or rod like shapes, well arranged within the polymeric matrix of proteins, mainly collagen type 1 fibrils, and polysaccharides (Mateus et al, 2008). Hydroxyapatite (HA) is a very complex crystalline compound with the sum-formula Ca_{10} (PO_4) $(OH)_2$, with the presence of carbonate ions, and possesses a high affinity for proteins (Roveri et al, 2008).
Different types of synthetic HA-NC are available for tissue engineering purposes, as injectable bone filling material contemporary able to deliver pharmaceutical molecules (Roveri et al, 2008).. Particularly, HA size is a relevant factor for in vitro cell activity, for bone substitution or for in vivo injectability. Moreover, injectable HA should have: *i)* fine and uniform particle size in the nanometer range; *ii)* phase homogeneity; *iii)* reduced degree of particle agglomeration (Ferraz et al, 2007). Instead, biomimetic HA-NC, showing low resorbability correlated to the crystalline structure and high tendence to aggregation, appears more useful when a biomaterial has to be implanted with a defined 3D form, as scaffold for bone growth. Many studies have demonstrated that HA-NC can be used also

for locally release of steroids, antibiotics, hormones and anticancer drug, as controlled drug delivery bone graft (Roveri et al, 2008).

Functionalisation can act as a strategy to fine-tune the bioactivity of HA and, recently, particular interest has been dedicated to the linking of bioconiugates, besides conjugation of drugs or other diagnostic/therapeutical agents.
Since HA has two different surface binding sites, called C and P, it contains multiple-site binding for proteins, gaining an high protein affinity. In fact, in virtues of its high protein affinity, a prime application of nano-phased HA is the purification of antibodies at small scale as well as industrial scale.
The fundamental studies of the absorption of acidic, neutral and basic proteins onto synthetic HA-NC have been carried out. It has been foregrounded as positive-charged sites, the protonated amino groups, the N-terminus and the amino group of lysine interact with the negatively charged HA phosphate group. The negatively charged residues, such as carboxylic groups of aspartate and glutamate, are interacting with the positively charged calcium ions in the HA. Also, phosphate groups and other negatively charged groups at the surface of the protein can contribute to the binding to HA (Jungbauer et al, 2004; Kamyshny et al, 1999; Vermeer et al, 2001).
Thus, the binding of proteins to the HA surface is mainly triggered by ionic interaction and the most important forces involved in the molecular adsorption can be expected to be electrostatic, associated with exposed charged group on protein surface (Kandori et al, 2005; Iafisco et al, 2005).
Besides HA features (size and morphology, specific surface area, ζ-potential - the overall charge that the particle acquires in a particular medium- and Ca/P ratio), factors determining the degree adsorption include protein conformation, isoelectric point (pI) and pH of buffers (Kandori et al, 2004; Jungbaeur et al, 2004).

On these basis, we decide to perform adsorption of immuno-gamma-globulins (IgG) onto HA-NC in order to carry out a preliminary study for the further development of a targeted drug delivery system, "disease-specific".
IgG or antibodies (M.W. 150 kD) are proteins synthesized by an animal in response to the presence of a foreign substance called an antigen. As figure 2 shows, an IgG is composed of four polypeptide chains, two heavy (M.W. 53 kD) and two light (M.W. 22.5 kD) chains, which form two Fab segments and one Fc segment with a "Y-shaped" conformation.

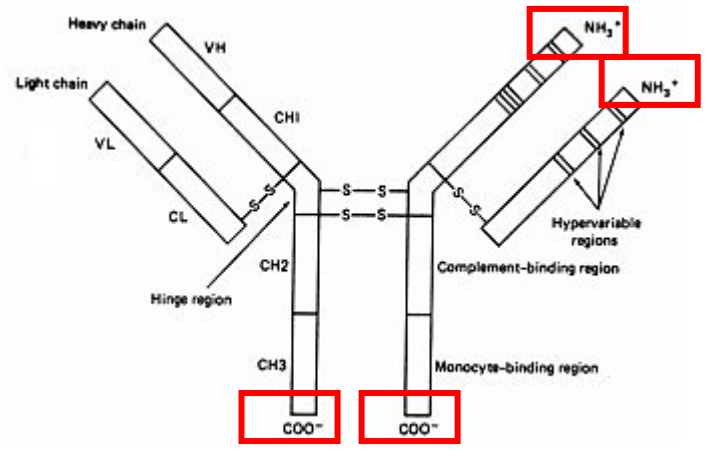


Fig. 2 Protein structure of immunoglobulin molecules (human IgG, 150 kD). It includes two light (L, 22.5 kD) and two heavy (H, 53 kD) chains, covalently linked by disulphide bonds between cysteine residues. The variable domains of the heavy chain (V_H) and the light chain (V_L) contribute to the binding site of the antibody molecule. The heavy-chain constant region is made up of three constant domains (C_H1, C_H2 and C_H3) and the (flexible) hinge region. The light chain also has a constant domain (C_L). Papain digestion subdivides molecule in: two "variable" F_{ab} fragment similar to each other and both showing a combinatory site for the antigen, whereas the third "constant" fragment, without antibody activity, is called F_c. Thus, the four N-terminal extremities are the variable regions, because of amino acids composing are different in type and sequence in each single IgG molecole. Invariable or constant region are instead represented by C-terminal extremities of light chain and by Fc fragment and they are responsible of IgG biologic activities.

Because of their strong binding affinity, they were applied for affinity chromatrography and high

performance liquid cromatrography (HPLC), immunoassay and biosensor systems. Many investigations have been already done using different adsorbents such as silica gel, polymer latex and Teflon particles (Kandori et al, 2004).
Previously, the adsorption of IgG onto HA has been reported only by few research groups. Rölla et al. identified IgA and IgG as part of the salivary layer on the tooth surface (Rolla et al, 1983; Eggen and Rolla, 1984). More recently, Kandori et colleagues presented adsorption data of IgG onto synthetic HA-NC. They compared the results obtained to those of acidic, neutral, and basic proteins, providing IgG onto HA-NC curves of kinetics and isotherm adsorption (Kandori et al, 2004). Finally, Ng et al, developing a model for study the absorption of protein onto HA-NC, showed that IgG binding was a function of calcium affinity interactions and cation exchange (Ng et al, 2007).

It is an attractive goal to create a new mimetic HA-NC for potential use in bone implantation, able to also represent a local targeted delivery system for drugs with controlled release properties (Jungbauer et al, 2004; Kandori et al 2005; Roveri et al, 2008). The final perspective is obtain "personalized medicines" with a significant reduction of injected dose and, in some cases, able to substitute natural tissue lost. Furthermore, inexpensive, but safe materials, as HA, have to be explored to reduce the cost of nanotechnology-based therapy, so that a greater patient population can benefits.
With continuous efforts by multidisciplinary team approaches, nanotechnology will display new pathway on diagnosis and therapy in cancer research.

Experimental Plan and Methods
Aim 1: HA-NC Synthesis and Characterization
The first goal was to prepare and characterize HA-NC with a nearly stoichiometric Ca/P ratio (5:3), in order to produce a plate-shaped bonelike apatite phase. HA-NC were grown, as previously described, at room temperature, by drop wise addition of ammonium phosphate to monohydrate calcium acetate solution, under stirring for 24 hours and adjusting pH to 10 with ammonium hydroxide (Bigi et al, 2009). HA-NC dimensions were determined using Transmission Electron Microscopy (TEM) images. The surface Ca/P ratio and crystallinity have been calculated by X-ray diffraction (XRD) technique at room temperature, which permits to identify the structure of apatite crystal. FT-IR (Fourier transform infrared) spectroscopy was performed to further characterize HA chemical structure, considering carbonates presence. Finally, specific surface area measurement was undertaken by using a Carlo Erba Sorpty 1750 instrument by measuring N2 adsorption onto apatite at 77 K and adopting the well-known BET procedure. ζ-potential was also calculated at pH 7.4.

Aim 2: Antibodies Immobilization on HA-NC
The general goal was to obtain samples of HA-NC with isothermal adsorbed polyclonal human IgG (Sigma Aldrich®). They were prepared by mixing 10 mg of HA with 1.5 mL of protein dissolved in Hepes buffer (0.01 M Hepes, 0.5 M NaCl) at pH 7.4 at four different concentrations (0.1-0.2-0.5-1 mg/mL) in a 2 mL conical polyethylene Eppendorf tube. The negative control was a similar tube only containing HEPES buffer.
Thus, samples were:

n. 10 mg HA in 1,5 mL HEPES buffer (negative control)
1. 1 mg/mL IgG + 10 mg HA in 1,5 mL HEPES buffer
2. 0.5 mg/mL IgG + 10 mg HA in 1,5 mL HEPES buffer
3. 0.2 mg/mL IgG + 10 mg HA in 1,5 mL HEPES buffer
4. 0.1 mg/mL IgG + 10 mg HA in 1,5 mL HEPES buffer

The mixture was maintained in a bascule bath at 37 °C for 24 h. The solids were recovered by centrifuging at 12 675*g* for 3 min and washed two times in HEPES buffer (0.01 M Hepes, 0.5 M NaCl) at pH 7.4, in order to eliminate all supernatants containing unbound IgG. All samples were stored at 4°C for further analysis.

Aim 3: Assessment and Quantification of Loaded Antibodies
Transmission electron microscopy (TEM) using immuno-gold labelling (colloidal gold conjugated to goat affinity purified antibody to human IgG) was performed to visually establish the presence of

adsorbed IgG. Samples number 1 (1 mg/mL IgG + 10 mg HA) and 2 (0.5 mg/mL IgG + 10 mg HA), besides the negative control, have been selected for the immuno-gold labelling assay. After defrosting, samples were left decanting for 3 hours, and centrifuged. 200 µl of their supernatant were taken off and replaced with fresh HEPES buffer (0.01 M, 0.05 M NaCl, pH=7.4). The samples were dispersed by a fast sonication (30 sec) and 10 µl of the slurries were deposited on holey-carbon foils copper micro-grids and left for 10 minutes, after what the excess solvent was removed by filter paper. Then 5 µl of the gold conjugate were put onto the grid and left interaction for 30 minutes. After that time, two washings by HEPES buffer were carried out directly onto the grid. The gold immuno-labelling assay was carried out in duplicate. Finally two washings by ultrapure water were performed, in order to remove the buffer salts from the grid. Then, they were dried at room temperature overnight. TEM analysis was conducted on a Jeol 1200 TEM operating at 120 keV.
In addition, to directly ascertain the presence of IgG loaded, a preliminary dot blot experiment was performed. From each sample, an amount (10 µl) of solids resuspended in HEPES buffer was collected: after the removal of surnatant, the IgG were eluted with the addition of TRIS HCl 1.5 M at pH 8.8, after a washing in glycine 100 mM at pH 3. Samples were then boiled in Laemmli buffer, in the presence β mercaptoethanol and then adsorbed onto Polyvinylidene Fluoride (PVDF) membrane. After blocking with 5% TBS/BSA, blots were probed with affinity-purified rabbit anti-human IgG antibodies, conjugated to rafan peroxidase. Chemiluminsescent reaction was performed applying the enhanced chemiluminescence system (ECL) system and PVDF membrane was read using Versadoc instrument.

Then, to indirectly measure the amount of IgG adsorbed on HA-NC, supernatants solutions, containing unbound IgG, were assayed for protein content by means of: *i)* UV vis spectroscopy (using an extinction of 1.45 $cm^3\ mg^{-1}$ at 280 nm); *ii)* applying spectrophotometric Bradford Protein Assay (at 595 nm); *iii)* performing a semi quantitative software analysis (Quantity One) on Comassie Blue 8% SDS PAGE gel, comparing samples with scalar amounts of BSA (2-1-0.5-0.250-0.125 µg). The quantity of adsorbed protein was indirectly calculated from the difference between the concentrations of the initial solution and the supernatant remained after conjugation.
Finally, Western Blot analysis (immuno-blotting) was used to directly quantify the loaded IgG, comparing samples with scalar amounts of IgG (0.5-0.250-0.125-0.062- 0.031 µg). From each sample, an amount (10 µl) of solids resuspended in HEPES buffer was collected: after the removal of supernatant, they were washed in glycine 100 mM (pH 3) and the supernatant boiled in Laemmli buffer, in the presence β mercaptoethanol. Then, they were subjected to SDS-PAGE and transferred to PVDF membrane by the high-intensity wet-blotting technique, as described previously. After blocking with 5% TBS/BSA, blots were probed with affinity-purified rabbit anti-human IgG antibodies, conjugated to horseradish peroxidase. Chemiluminsescent reaction was performed applying the enhanced chemiluminescence system (ECL) and PVDF membrane was read using Versadoc instrument. Semi-quantitative densitometric quantification report was provided by Quantity One software analysis.

Aim 4: Stability of the complex HA-NC-Antibodies

The final step of the project was to verify the stability of the link between NPs and IgG, evaluating the amount of IgG released into supernatant. After conjugation, washed samples were preserved at 4°C, as previously described. At 1^{st}, 2^{nd}, 3^{th}, 4^{th}, 5^{th}, 7^{th} weeks from immobilization, an amount from each one was collected from supernatant, subsequent to centrifuging at 12 675*g* for 3 min.
The amounts of IgG released were directly assayed, week per week, for protein content by UV vis spectroscopy (at 280 nm); then, after having collected each week an amount of 30 µl from samples, Western blot analysis were performed, using the same procedure beforehand reported. For Western Blot investigation, only the following samples were selected:

1. sample release 1 of the 1^{st} week (1 mg/mL IgG + 10 mg HA)
2. sample release 1 of the 2^{nd} week (1 mg/mL IgG + 10 mg HA)
3. sample release 1 of the 3^{th} week (1 mg/mL IgG + 10 mg HA)
4. sample release 1 of the 4^{th} week (1 mg/mL IgG + 10 mg HA)
5. sample release 1 of the 5^{th} week (1 mg/mL IgG + 10 mg HA)
6. sample release 1 of the 7^{th} week (1 mg/mL IgG + 10 mg HA)

7. sample release 2 of the 7 th week (0.5 mg/mL IgG + 10 mg HA)
8. sample release 3 of the 7 th week (0.2 mg/mL IgG + 10 mg HA)
9. sample release 4 of the 7 th week (0.1 mg/mL IgG + 10 mg HA)
10. sample release 5 of the 7 th week (10 mg HA)

Results

HA-NC Characterization

A bonelike plate-shaped HA was obtained, showing features (size, crystallinity and Ca/P ration) to very similar natural bone.

TEM images (i.e. figure 3) demonstrate length dimensions ranging between 10±5 nm and they clearly depict HA-NC high aggregation rate, also supported by ζ-potential value, calculated as – 0.5 mV. The magnitude of the measured ζ-potential is an indication of the repulsive force that is present: if all the particles in suspension have a large negative or positive ζ-potential, they will tend to repel each other and there is no tendency for the particles to come together. If the particles have low ζ- potential values, as in this case because of synthesised HA-NC, there is no force to prevent the particles coming together.

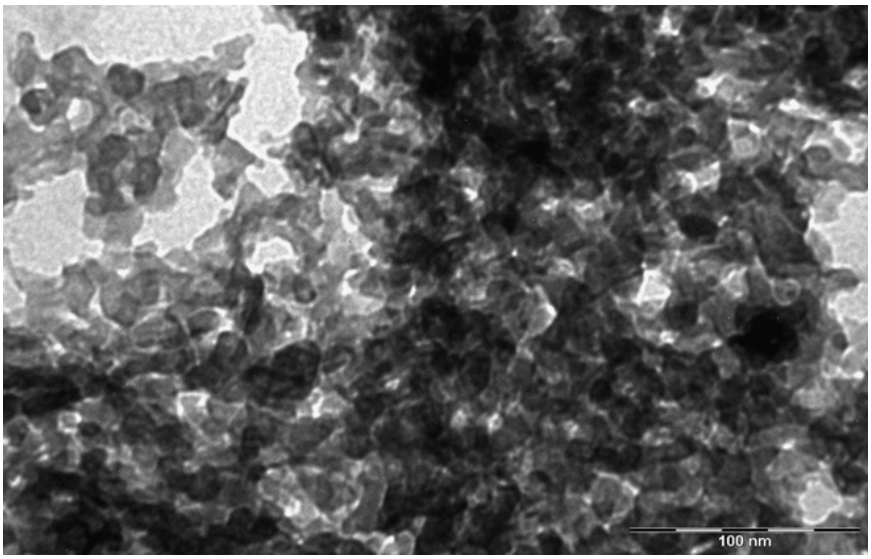


Fig.3 TEM image depicts bonelike HA-NC**,** obtained through drop-wise reaction: hexagonal plate-shaped apatite, with high aggregation rate.

In figure 4, XRD curve is reported, revealing the typical apatite phase pattern with a low degree of crystallinity (30±4 %) and a surface Ca/P ratio of 1.45±0.05.

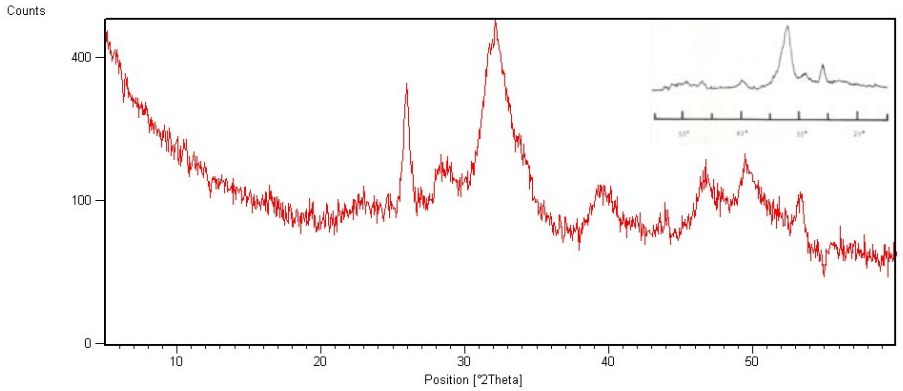


Fig.4 XRD analysis illustrates synthesized bonelike HA-NC: it is depicted the typical pattern of apatite phase, with peaks at about 25.9 and 31-33 θ. Synthesized HA-NC (in red) has a similar curve to natural bone HA-NC (upper, in black). The graphic represents typical peaks of HA-NC, plate-shaped and hexagonal form, with low rate of crystallinity and a Ca/P ratio of about 5:3, similar features to those of bone.

Synthesised HA was B type, as showed by FT-IR description: during reaction, carbonates replaced some phosphates in apatite phase (figure 5).

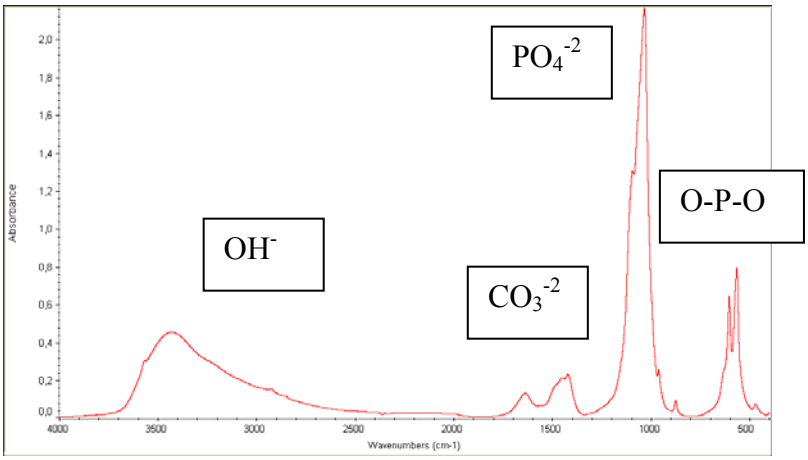
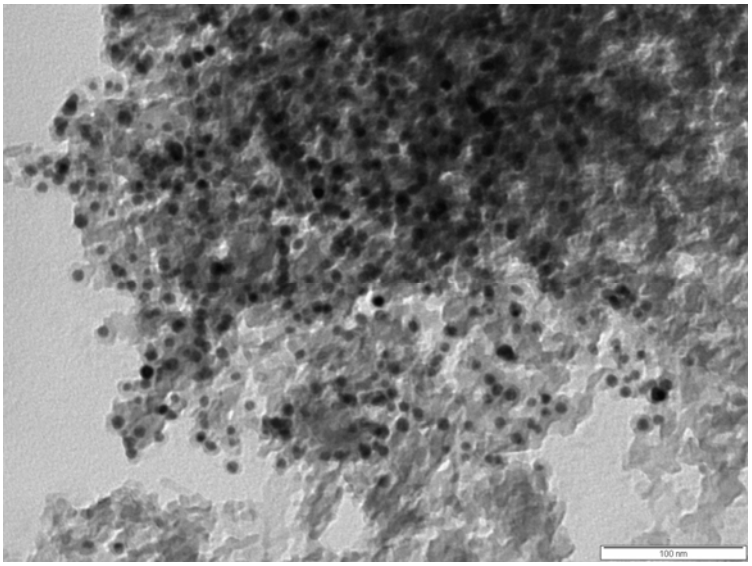


Fig.5 FT-IR image described chemical composition of HA-NC. In this case, HA attained is B type, where carbonate ions have been introduced and substituted to phosphates, in order to obtain this kind of HA, similar to that found in bone.

BET analysis was performed to ascertain the specific surface area of HA-NC: it was of 120± 6m^2/g.

Assessment and Quantification of Loaded Antibodies

TEM images of biomimetic HA-NC interacting with IgG are shown in figures 5 a, b, c. Gold immuno-labelling is more present in figures 6 a and b, referring to sample 1 and 2 respectively (1 mg/mL IgG + 10 mg HA; 0.5 mg/mL IgG + 10 mg HA). This suggest a clear presence of IgG onto HA surface. Gold immuno-labelling can be also found in figure 6 c, which corresponds the negative control (10 mg HA in 1,5 mL HEPES buffer). In all samples, HA seems to aggregate.



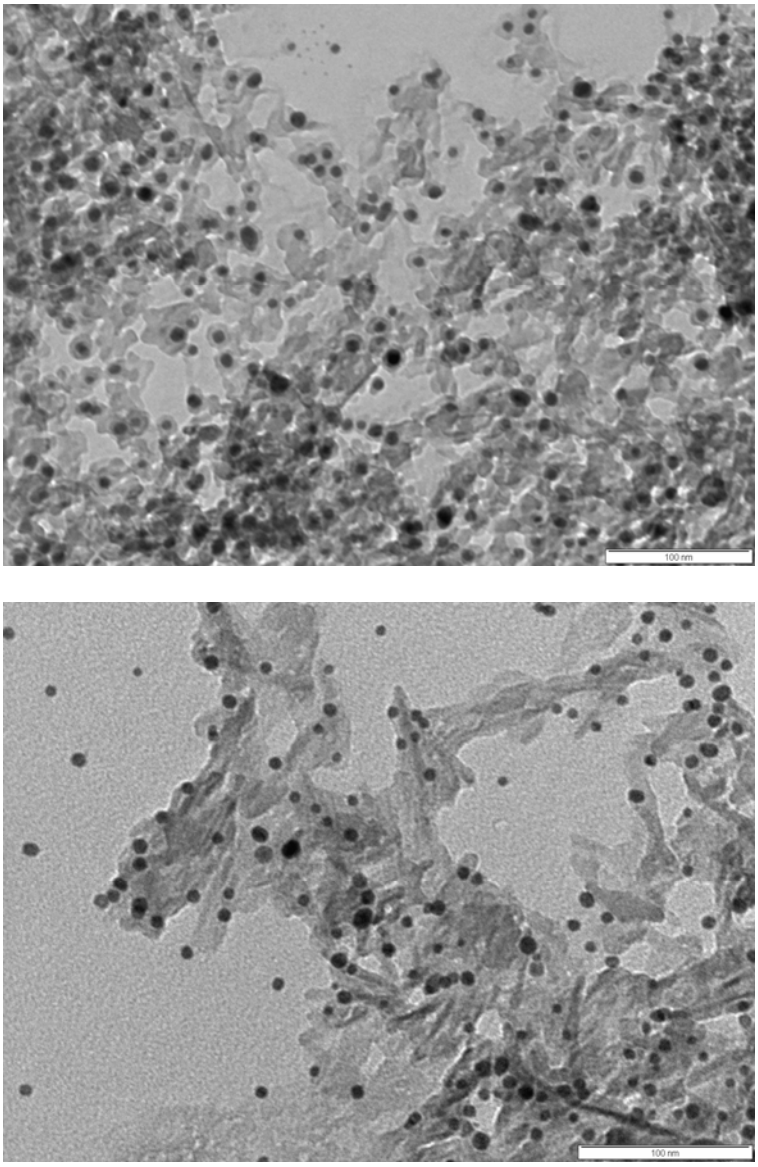


Fig.6 a- TEM images of sample 1 (1mg/mL + 10 mg HA): IgG adsorbed onto biomimetic HA-NC are identified using gold immuno-labelling **b-** TEM images of sample 2 (0.5 mg/m + 10 mg HA): IgG adsorbed onto biomimetic HA-NC are identified using gold immuno-labelling **c-** TEM images of sample 5 (10 mg HA in 1,5 mL HEPES buffer, negative control): IgG adsorbed onto biomimetic HA-NC are identified using gold immuno-labelling **.**

Despite proving evidence of IgG presence, TEM images showed positivity also for negative control, because of the high affinity of HA-NC for proteins: gold labelled goat anti human IgG has been adsorbed in the same way of the human IgG utilised for this study, being the immobilization reaction a simple incubation at room temperature. However, a clear visual quantitative difference can be foregrounded. In addition, TEM photographs point out HA-NC aggregation, even in presence of IgG (with low aggregation factor), which, thus, seem not able to give any modification on HA-NC ζ-potential, as instead previously depicted for myoglobulin (Iafisco et al, 2007; Kamyshny et al, 1999).
Dot blot (figure 7) confirms the presence of IgG onto HA-NC and provides preliminary data for the further Western Blot analysis: the first four spots represent samples 1, 2, 3, 4 (1 mg/mL IgG + 10 mg HA; 0.5 mg/mL IgG + 10 mg HA; 0.2 mg/mL IgG + 10 mg HA; 0.1 mg/mL IgG + 10 mg HA), where positivity of the experiment is related to the their darker circular outline; the last one spot is the negative control (10 mg HA in 1,5 mL HEPES buffer), where the black margin is lacking.

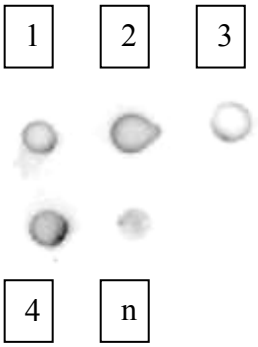


Fig. 7 Dot Blot analysis permits to ascertain IgG absorption onto HA-NC. Each spot represents one of the five samples:

5. 1 mg/mL IgG + 10 mg HA in 1,5 mL HEPES buffer
6. 0.5 mg/mL IgG + 10 mg HA in 1,5 mL HEPES buffer
7. 0.2 mg/mL IgG + 10 mg HA in 1,5 mL HEPES buffer
8. 0.1 mg/mL IgG + 10 mg HA in 1,5 mL HEPES buffer

n. 10 mg HA in 1,5 mL HEPES buffer (negative control)

The indirect quantification of IgG adsorbed onto HA-NC, performed by spectrophotometric assay at 280 nm and Bradford Method, permits to calculate the isotherm absorption curve, which correlate the amount of unbound IgG to the "coverage" (Γ_{IgG} mg/m^3). The latter is the ratio between the total adsorbed protein and NPs specific surface area (previously found as 120±6 m^2/g). The two absorption curves are similar, as shown in figures 8 and 9.

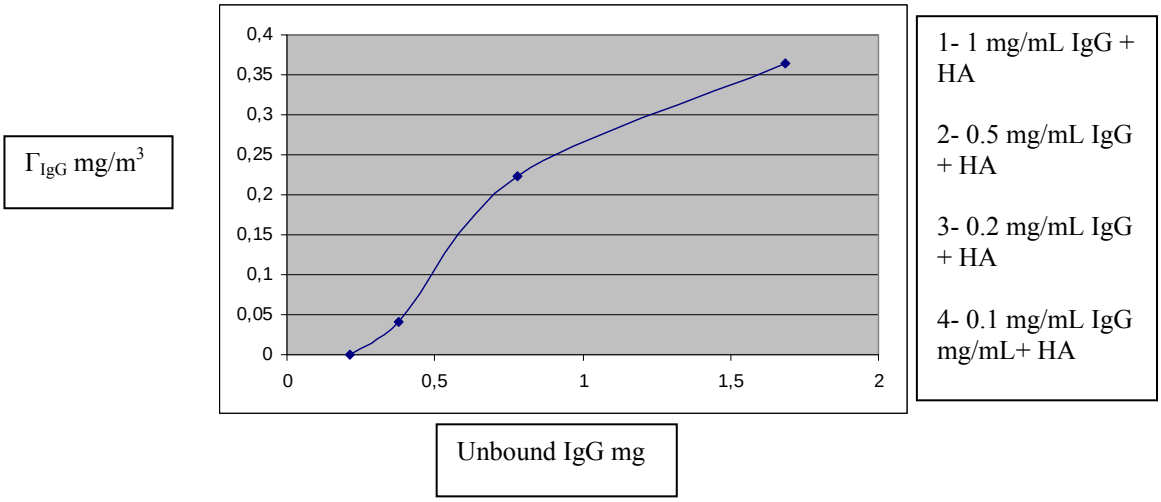


Fig. 8 IgG adsorption onto HA-NC at pH 7.4 has been evaluated using Bradford Method. The graphic shows isotherm absorption curve IgG onto HA, which creates a correlation between the amount of unbound IgG and the ratio adsorbed IgG/surface area ratio.

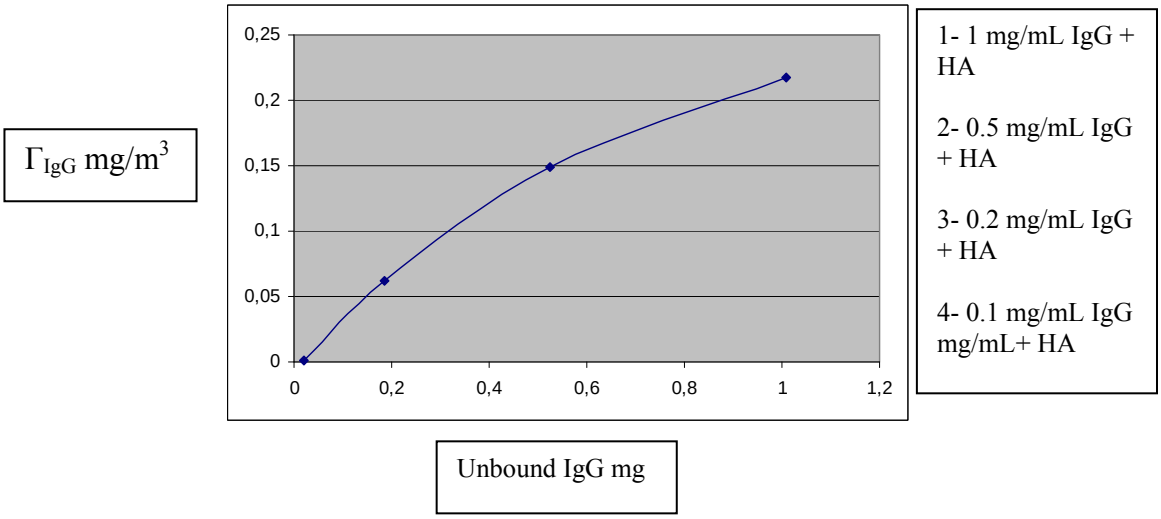


Fig. 9 IgG adsorption onto HA-NC at pH 7.4 has been evaluated using UV vis spectrophotometer (280 nm). The graphic shows isotherm absorption curve IgG onto HA, which creates a correlation between the amount of unbound IgG and the ratio adsorbed IgG/surface area ratio.

The simple ratio between adsorbed IgG and the original antibodies amount utilized for immobilization reaction (loaded IgG/initial IgG) suggests that, for samples 2 and 3 (0.5 mg/mL IgG + 10 mg HA; 0.2 mg/mL IgG + 10 mg HA), 1/3 of the total IgG were adsorbed, 1/4 for sample 1 (1 mg/mL IgG + 10 mg HA) and 1/7 for sample 4 (0.1 mg/mL IgG + 10 mg HA).
These data are confirmed by the semi-quantitative investigation, applying a densitometric software analysis on the Comassie Blue SDS PAGE gel (figure 10 a). The first five wells hold scalar BSA amounts (2-1-0.5-0.25-0.125 μg), while the following ones are occupied by the five supernatant samples, 1, 2, 3, 4, n, respectively.
Isothermal absorption curve (figure 10 b) is less specific and slightly different from the others, even if the ratio between adsorbed IgG and initial IgG amounts is maintained.

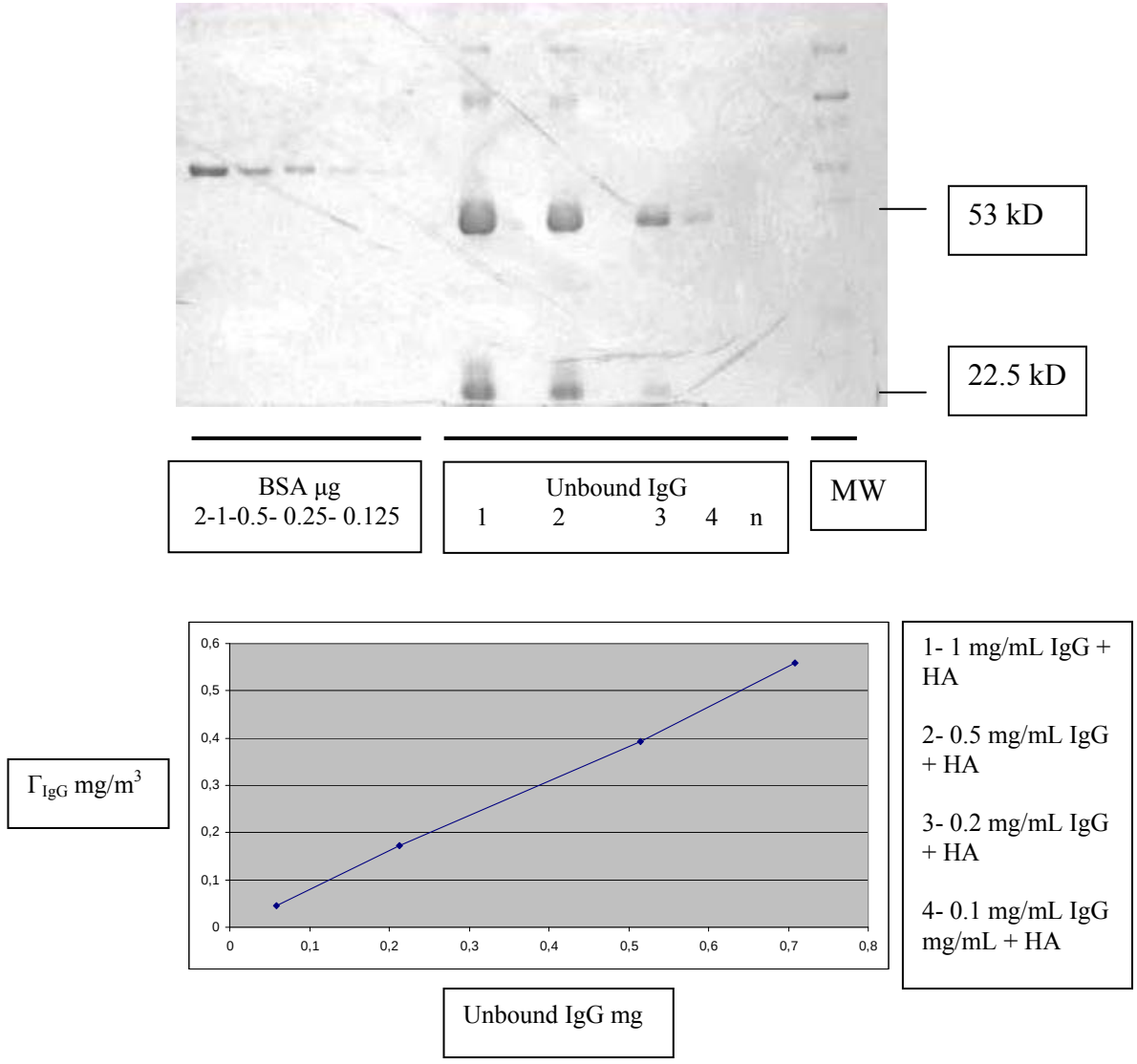


Fig. 10 a- Gel SDS-PAGE has been performed for quantification of IgG unbound (1- 1 mg/mL IgG + 10 mg HA; 2- 0.5 mg/mL IgG + 10 mg HA; 3- 0.2 mg/mL IgG + 10 mg HA; 4- 0.1 mg/mL IgG + 10 mg HA). Each surnatant was compared to scalar BSA amounts (2-1-0.5- 0.25- 0.125 μg), both visually and through densitometric software analysis (Quantity One) **b-** IgG adsorption onto HA-NC at pH 7.4 has been evaluated using semiquantitative analysis performed on Blue di Comassie gel (Quantity One). The graphic shows isotherm absorption curve IgG onto HA, which creates a correlation between the amount of unbound IgG and the ratio adsorbed IgG/ surface area ratio.

Furthermore, a densitometric Western Blot investigation of adsorbed IgG (compared to scalar amount of IgG) was carried out. In figure 11, the first five wells represent scalar IgG amounts (0.5-0.250-

0.125-0.062-0.031 μg), whereas the following five contain each of the five samples, n, 1, 2, 3, 4 respectively.
Both visually and by Quantity One software analysis, no correspondence between adsorbed IgG and initial IgG amounts is found.

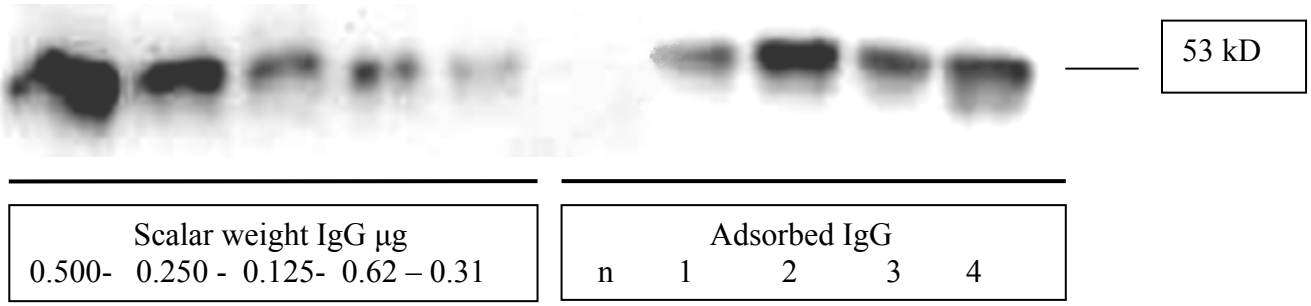


Fig.11 Western Blot analysis has been carried out to quantify IgG loaded (n- 10 mg HA in 1,5 mL HEPES buffer onto HA-NC; 1- 1 mg/mL IgG + 10 mg HA; 2- 0.5 mg/mL IgG + 10 mg HA; 3- 0.2 mg/mL IgG + 10 mg HA; 4- 0.1 mg/mL IgG + 10 mg HA). Each sample was compared to scalar IgG amounts (0.5-0.25-0.125-0.62-0.31 μg), both visually and through densitometric analysis by Quantity One software.

Therefore, this study corroborates isothermal adsorption of IgG onto synthetic HA-NC, as reported in only a previous study by Kandori and colleagues (Kandori et al, 2004; Ng et al, 2007), even if it was not confirmed from densitometric Western Blot analysis. This is probably due to the difficulty to collect the same amount of IgG-HA-NC complexes. In fact, HA-NC form a no homogeneous solution, showing high degree of precipitation.
The binding of IgG onto the HA surface has been verified and, on the basis of literature, it is expected to be mainly triggered by ionic interaction and governed by electrostatic and Van der Waals interactions, associated with exposed charged group on protein surface, as already described (Iafisco et al, 2005; Haupt et al, 1995). Moreover, principal variables, which can regulate absorption, are related to: *i)* HA-NC (ζ potential; Ca/P ratio; surface area); *ii)* IgG population (pI, factor of aggregation, solubility limit); *iii)* pH buffer (Geoghegan, 1988; Kandori et al, 2004). Besides HA features, mainly pI of IgG population and pH of the buffer used play important roles. Even if serum IgG represents the most isoelectrically heterogeneous protein, Haupt at al. reported that the optimal antibodies absorption condition was using TRIS-HCl pH 8.5 (pI 8.2-9.3) or HEPES buffer pH 7 (pI 7.0-9.3). We used the latter because it permits to lead the experiment using a pH closed to that physiologic. At these conditions, since IgG seem to have a neutral/basic pI and slight positive net charge, as well as myoglobin, similar absorption behaviour can be expected on both proteins. Instead, as Kandori and colleagues already depicted, the isotherms and kinetics curves were close to those of the acidic protein lysozime, indicating that the values are much less correlated with the HA C-sites (Kandori et al, 2004).
Moreover, these results foreground a relation between the quantity of IgG added and the absorption effect, as already suggested by Gheoghegan (Gheoghegan, 1988). In fact, quantification data point out that sample 1, obtained by mixing HA-NC with the major amount of antibodies (1mg/mL) shows a decrease of the amount of adsorbed IgG (1/4). This can be explained by the competition between proteins themselves in order to gain an absorption site onto HA-NC, particularly considering that the upper concentration we utilised for immobilization (1mg/mL) was close to achieve absorption saturation point, reached at about 1,5 mg/mL as already described (Kandori et al, 2004).

Stability of the complex HA-NC-Antibodies

Being all samples stored at 4°C, the potential amounts of IgG released into the supernatant were preliminary assayed by UV vis spectroscopy (at 280 nm), at 1^{st},2^{nd},3^{th},4^{th},5^{th},7^{th} weeks from the conjugation reaction: all data were negative.
Western blot analysis on some selected release samples was carried out. The figure 12 reveals a steady bound of IgG onto HA-NC. Only weak signals are detected in correspondence of release sample 1 (1 mg/mL IgG + 10 mg HA) within first two weeks, whereas no unbound IgG are spotted on the following weeks.

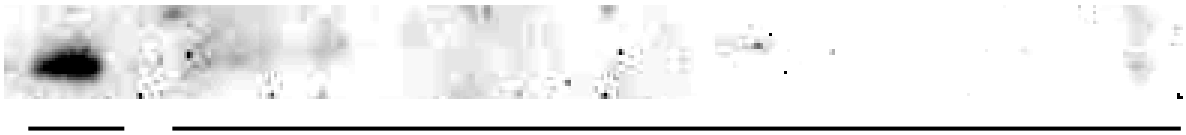


Fig.12 Western Blot analysis for evaluating IgG released from HA-NC. The experiment was performed on the following selected samples:

11. release of sample 1 at 1st week (1 mg/mL IgG + 10 mg HA)
12. release of sample 1 at 2nd week (1 mg/mL IgG + 10 mg HA)
13. release of sample 1 at 3th week (1 mg/mL IgG + 10 mg HA)
14. release of sample 1 at 4 th week (1 mg/mL IgG + 10 mg HA)
15. release of sample 1 at 5 th week (1 mg/mL IgG + 10 mg HA)
16. release of sample 1 at 7 th week (1 mg/mL IgG + 10 mg HA)
17. release of sample 1 at 8 th week (1 mg/mL IgG + 10 mg HA)
18. release of sample 2 at 7 th week (0.5 mg/mL IgG + 10 mg HA)
19. release of sample 3 at 7 th week (0.2 mg/mL IgG + 10 mg HA)
20. release of sample 4 at 7 th week (0.1 mg/mL IgG + 10 mg HA)
21. release of sample 5 at 7 th week (10 mg HA)

Confirming the high structural stability related to the isotherms curves proposed by Kandori et al, data concerning IgG release during the weeks are provided: no desorbed IgG are revealed, underlining a stable bind which is probably the sum of several electrostatic links. Adsorbed IgG, showing weaker bound are released within the first two weeks from the immobilization reaction.
In addition, it has also reported that adsorbed IgG molecules become more tightly as the contact time with adsorbent surface increase (Kandori et al, 2004).

These results represent progress in the first years of my Ph.D. program, in which I became familiar with protein-HA-NC isothermal adsorption reaction, spectrophotometrical protein assay, Blue di Comassie gel SDS Page and Western Blot analysis.
The use of biomimetic HA resulted very promising in orthopaedics, dental implantology and drug delivery, due to its composition, structure, dimension and morphology similar to those of natural bone crystals and to its ability to bind pharmaceuticals agents as well as peptides and proteins.
In the development of therapeutic and/or diagnostic reagents, the main goal is to realize firm attachment of IgG molecules at the solid surfaces without interrupting or covering the biological functions of the protein, such as selectivity and specificity. Because the antigen-binding sites are located on the far ends of the F_{ab} segments, it is desired that IgG molecules absorption takes place onto HA-NC surface by anchoring F_c parts, with exposing the variable F_{ab} segments toward to the outside.
Therefore, these findings indicate that we have obtained suitable bioconiugates for further studies. If antibodies stable bind and correct configuration will be achieved, bonelike HA-NC can be considered an important tool in nanomedicine both as targeted drug delivery system and as bone graft.
We are now preparing to initiate similar experiments using monoclonal antibody against a receptor overexpressed in some cancer cells in order to produce bi-functionalised NPs and evaluate hypothetical interference between drug and antibodies absorption/activity.

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Stage presso enti esterni nell'anno accademico 2008-09

Gli "stage" si intendono come periodi di apprendistato lavorativo e/o di ricerca presso enti/aziende italiane o straniere. Ogni studente deve compilare le schede rispettando il formato (Times New Roman 12) e l'impaginazione in quanto i dati saranno inclusi come tali nelle schede da inviare al nucleo di valutazione dell'Ateneo.

Studente	Ciclo	Ente presso cui si è svolto lo stage	Periodo [in mesi]	Argomento
ELENA M. VARONI	XXIV	Ospedale San Paolo – Unità Di Medicina e Patologia Orale Università degli studi di Milano	6 mesi	Medico frequentatore

{Aggiungere le righe necessarie}

Percorso formativo nell'anno accademico 2008-09

Lo studente deve elencare tutti i corsi didattici, workshop e seminari ai quali ha partecipato nell'anno accademico 2008-2009. Rispettare il formato e l'impaginazione come sopra.

Corso di insegnamento, workshop o seminario (+ località)	Docente (+ Affiliazione)	Numero di ore	
Lezione introduttiva II - Immunopatologia	Prof. Umberto Dianzani (Univ. del Piemonte Orientale)	11-11-2009	Firme di frequenza
Journal Club (Novara)	Prof.ssa Mara Giordano (Univ. del Piemonte Orientale)		Firme di frequenza
Production of bio-farmaceuticals in plants	Prof. Luca Santi (Univ. degli Studi di Roma "Tor Vergata")	5-12-2008	
Le cellule Mesenchimali Staminali per il trattamento delle malattie neurologiche	Prof. Antonio Uccelli (Univ. degli Studi di Genova)	12-12-2008	
La malattia celiaca	Prof. Riccardo Troncone (Univ. di Napoli "Federico II")	9-01-2009	
Non solo motoneuroni: il contributo del muscolo scheletrico alla patogenesi della sclerosi laterale amiotrofica	Prof. Antonio Musarò (Univ. di Roma "La sapienza")	10-02-2009	
The use of cytomegalovirus as a novel vaccine vector for Simian Immunodeficiency Virus	Prof. J. Nelson (Vaccine & Gene Therapy Institute Oregon Health & Science University)	19-02-2009	

When a fine tuner of signaling pathways becomes a dangerous amplifier: > the case of the Prolyl-isomerase Pin1 in cancer	Prof. Giannino Del Sal (Univ. di Trieste)	20-02-2009	
Giornata di Microscopia Confocale	Dipartimento di Anatomia e Morfologia Umana (Univ. degli Studi di Milano)	4-03-2009	Attestato di partecipazione
La proteomica: un nuovo modo di guardare al mondo delle proteine	Prof. Piero Pucci (Univ. di Napoli Federico II)	18-03-2009	
Le nanotecnologie applicate alla medicina	Prof. G. Peluso (CNR di Napoli)	19-03-2009	
Regulation of integrin functions in vascular systems	Prof. Federico Bussolino (IRCC di Candiolo-Torino)	26-03-2009	
Corso "Fondamenti di preparativa ed osservazione in immunoistochimica ed immunofluorescenza"	Dipartimento di Anatomia e Morfologia Umana, Prof. Isabella Barajon (Univ. degli Studi di Milano)	Dal 30-03-2009 al 3-04-2009	Attestato di partecipazione
Neurotossicità delle proteine 'misfolded' nelle malattie del motoneurone	Prof. Angelo Poletti, Univ. di Milano	12-05-2009	
Functional Genomics of Brain	Prof. Stefano Gustincich (SISSA di Trieste)	14-05-2009	
Role of Raf in tumorigenesis	Prof. Baccarini (Max F. Perutz Laboratories of Vienna)	03-06-2009	

{Aggiungere le righe necessarie}

Nelle tabelle, i valori richiesti si riferiscono alla **carriera complessiva del Dottorando**. Ogni studente deve inserire i dati richiesti relativi al ciclo di appartenenza. Rispettate formato ed impaginazione

Tabella C.1 - Studenti iscritti

Ciclo XXIV

Nome e Cognome	Anno nascita	Laureato/a presso Università di …	Borsa (SI/NO)	Se senza borsa, forma di sostegno economico	Mesi totali trascorsi all'estero	Numero pubblicazioni	Numero di partecipazioni a conferenze
ELENA MARIA VARONI	**1984**	**Milano**	**SI**		**0**	**4**	**1**

{Aggiungere le righe necessarie}

(1) Allegare l'elenco dei **soggiorni**

Studente	Ente	Luogo	Anno	Docente/ricercatore di riferimento nell'ente ospitante (*)
ELENA M. VARONI	Università di Bologna Laboratorio di Chimica Strutturale Biologica e dell'Ambiente	Bologna	2009	Dott. Michele Iafisco

(*)Se applicabile.

(2) Allegare l'elenco delle **pubblicazioni (**di cui almeno un dottorando è co-autore)

VARONI E., A. MOLTENI, G. LODI, I. ANGELI, F. GIGLI, D. DI CANDIA, F. LODI (2008). Topical clobetasol: is its systemic absorption real? A preliminary study about the application on oral mucous. In: Oral Diseases. Salzburg, Austria, 18-20 September 2008 Wiley-Blackwell, vol. 14 (supplement 1), p. 28, ISBN/ISSN: 1354-523X

MOLTENI A, VARONI E., DEMAROSI F, DI CANDIA D, LODI F, LODI G (2009). Clobetasolo topico ed assorbimento sistemico: studio preliminare dopo applicazione sulla mucosa orale. In: Atti Congresso Nazionale dei Docenti di Discipline Odontostomatologiche e Chirurgia Maxillo Facciale. Roma, 22-24 Aprile 2009, ROMA

FEDERIGHI V, VARONI E., SARDELLA A, DEMAROSI F, LODI G (2009). Peso specifico della saliva totale basale raccolta mediante spitting method. In: Atti Congresso Nazionale dei Docenti di Discipline Odontostomatologiche e Chirurgia Maxillo Facciale. Roma, 22-24 Aprile 2009, ROMA

VARONI E., FEDERIGHI V, LUDWIG N, GARGANO M, PANDINI D, CARASSI A (2009). Studio preliminare sul colore in odontoiatria conservativa mediante spettrofotometro "HR4000" con sonda a fibre ottiche. In: Atti Congresso Nazionale dei Docenti di Discipline Odontostomatologiche e Chirurgia Maxillo Facciale. Roma, 22-24 Aprile 2009, ROMA

(3) Allegare l'elenco delle **conferenze/workshop (**a cui almeno un dottorando ha partecipato)

Studente	**Conferenza/Workshop**	**Luogo**	**Anno**	**Studente relatore** {SI, NO}
ELENA M. VARONI	Congresso Nazionale dei Docenti di Discipline Odontostomatologiche e Chirurgia Maxillo Facciale	Roma	2009	No

Ciclo XXIII

Nome e Cognome	**Anno nascita**	**Laureato/a presso Università di …**	**Borsa (SI/NO)**	**Se senza borsa, forma di sostegno economico**	**Mesi totali trascorsi all'estero**	**Numero pubblicazioni**	**Numero di partecipazioni a conferenze**

{Aggiungere le righe necessarie}

(1) Allegare l'elenco dei **soggiorni**

Studente	Ente	Luogo	Anno	Docente/ricercatore di riferimento nell'ente ospitante (*)

(*)Se applicabile.

(2) Allegare l'elenco delle **pubblicazioni (**di cui almeno un dottorando è co-autore)

(3) Allegare l'elenco delle **conferenze/workshop (**a cui almeno un dottorando ha partecipato)

Studente	Conferenza/Workshop	Luogo	Anno	Studente relatore {SI, NO}

Ciclo XXII

Nome e Cognome	Anno nascita	Laureato/a presso Università di …	Borsa (SI/NO)	Se senza borsa, forma di sostegno economico	Mesi totali trascorsi all'estero	Numero pubblicazioni	Numero di partecipazioni a conferenze

{Aggiungere le righe necessarie}

(1) Allegare l'elenco dei **soggiorni**

Studente	Ente	Luogo	Anno	Docente/ricercatore di riferimento nell'ente ospitante (*)

(*)Se applicabile.

(2) Allegare l'elenco delle **pubblicazioni (**di cui almeno un dottorando è co-autore)

(3) Allegare l'elenco delle **conferenze/workshop (**a cui almeno un dottorando ha partecipato)

Studente	Conferenza/Workshop	Luogo	Anno	Studente relatore {SI, NO}