

Studies concerning the chemical immobilization of dendrimers on macroporous polymer matrix

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A highly innovative combination of dendrimers with non-biodegradable host polymers (poly(2-hydroxyethyl methacrylate)) (PHEMA) was researched in order to obtain a completely new approach to bone regeneration. This study was aimed at the development of hybrid materials Polymer (PHEMA) - Polyamidoamine dendrimers (PAMAM), as smart biomaterials with Hydroxyapatite (HA) forming ability. Besides the capacity to form HA when implanted, these hybrids are designed with special macroporous interconnected architecture similar to that characteristic to the trabecular bone. The success of the binding of PAMAM on the surface of PHEMA matrix was checked through FTIR analyses. The hybrids will be further characterized and *in vitro* evaluated with respect to their capacity to initiate HA formation when used as bone grafts with osteoconductive properties.

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1. Introduction

Current treatments of large bone defects mainly focus on the reconstruction of the defect using biomaterials with osteoinductive, osteoconductive or filling properties [1-4]. Polymeric materials which allow mineral formation are very important in orthopedics because their *in situ* transformation in composites polymer-HA similar to the bone natural ones. Compared to the synthetic composites, the *in situ* HA-forming ones offer a more suitable biological and durable solution for bone repair based on the patient's own tissue.

Mineralization of a synthetic or natural matrix is a complex phenomenon. There are numerous factors deciding the mineralization occurrence, but the presence of calcium and phosphate ions in the proximity of the implant represents the *sine qua non* condition. Calcium ions are provided by the surrounding cells and circulating fluids. Phosphate ions are supplied by the hydrolysis of phosphoesters or phosphoproteins, from circulating ions and/or from the breakdown of pyrophosphate [5]. Negatively and positively charged groups at physiological pH have well known potential as heterogeneous nucleation of HA through calcium and phosphate ions capturing from the surrounding fluids [2,3,6-13].

Starting from the idea that defined chemical functionality can decide on the nucleation and crystal growth of inorganic materials, *dendrimers and hyper-branched polymers* have been proposed and researched as *templates for the formation of biominerals*, mainly leading to materials with well defined shapes and properties [14]. This study was aimed at the development of new hybrid materials combining a macroporous polymer matrix consisting in PHEMA with the mineralization potential of globular dendrimers with different functionality (see Fig. 1).

Dendrimers are nanometer-sized macromolecules, with tree-like or star-like structure, containing an important number of terminal groups that can be modified to suit a specific application. Several dendritic structures are commercially available; among them, PAMAM class represents by far the most extensively researched for biomedical applications [15-17]. They are available under a wide range of functional ending-groups appropriate to specific modifications.

All these considerations made us to choose PAMAM dendrimers as further templates for HA formation on the surface of the polymeric material. Different generations of PAMAM with -NH₂ and -COOH functional end-groups were used.

PHEMA was selected as hybrid matrix due to its biocompatibility and suitability for hard tissue replacement applications as well as for its bone filling well-known potential.

The work followed two main directions: 1) the synthesis of a macroporous PHEMA matrix with interconnected open porosity; 2) the chemical binding of globular dendrimers on the surface of PHEMA porous scaffolds.

This communication reports on the modification of the PHEMA surface with dendritic molecules. The evaluation of the success of the reaction was performed by infrared spectroscopy.

2. Experimental

Materials

The monomer 2-hydroxyethyl methacrylate (HEMA) was used after distillation under reduced atmosphere. Benzoyl peroxide (BPO) and N,N-dimethyl-p-toluidine (NNDMpT) were used to initiate the polymerization. Poly(methyl methacrylate) (PMMA) beads with average

diameter around 250 μ (prepared in our laboratory) were used as pores generators.

PAMAM dendrimers were supplied from Sigma-Aldrich and used as received, as solutions in methanol. Generations G1.5 and G3.5 were used for the dendrimers with -COOH functional end-groups. G2 and G4 were used for the -NH₂ ending dendrimers.

All the reagents and the solvents used in the study were supplied from Sigma-Aldrich.

Methods

Polymer matrix: The synthesis of the polymeric scaffold is schematically presented in Fig. 2. The procedure is based on a previously reported protocol [18]. The two steps procedure consists in 1) the polymerization around the porogen beads followed by 2) the removal of the porogen with pores creation inside the matrix.

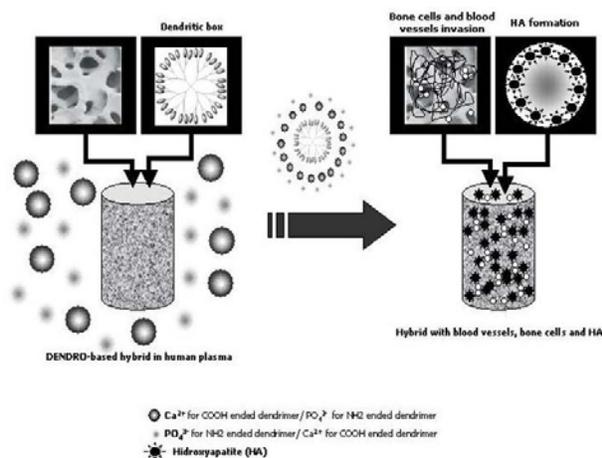


Fig. 1. Schematic view of how the bone regenerative hybrids will work.

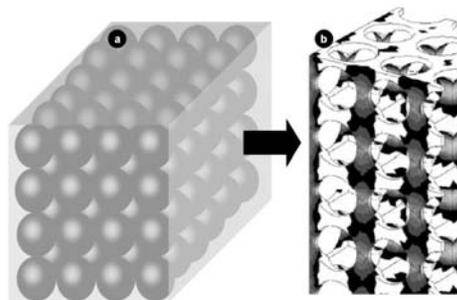


Fig. 2. Synthesis of porous polymer.

1) The polymerization mixture consisting in BPO dissolved in HEMA (1% molar ratio) and NNDMPt (1:1 to BPO) is degassed (nitrogen bubbling) and poured in 2 ml plastic tubes containing the PMMA beads. The beads weight should be previously determined (m_0). The polymerization occurs at room temperature and can be considered achieved after 6 hours. The PHEMA blocks containing PMMA beads inside them (Figure2 a) were dried in an oven, and then weighted (m_1).

2) The porogen was removed by extensive extraction in dichloroethane. After 48h the porous blocks (Figure2 b) were dried and weighted (m_2).

The complete removal of the porogen was checked by comparing (m_1-m_2) compared with m_0 .

The synthesis of the hybrid material:

The dendrimers were immobilized on the PHEMA matrix following a two steps procedure consisting in: 1) the chlorination of the polymer with SOCl₂ (in carbon tetrachloride, catalyzed by pyridine, at room temperature) [19] and 2) the chemical immobilization of the dendrimers (different generations) carrying -COOH or -NH₂ surface groups on the already chlorinated polymeric matrix [20]. The chlorinated macroporous PHEMA scaffolds were immersed in a methanolic solution containing the dendrimers. The surface modification was considered achieved after 24 hours, at room temperature. Theoretical 1:100 molar ratios between functional ending groups of dendrimers and chlorinated HEMA units were used. The reaction was expected to occur only at the surface of the porous blocks.

Characterization:

The hybrid materials were morphologically evaluated by light microscopy (ZEISS, Axiotech). A model of porosity detailed evaluation could be explored by scanning electron microscopy and computed microtomography, as we have already reported elsewhere [18].

Infrared spectra of the powdered samples compressed in KBr discs were recorded on a Fourier transform infrared spectrometer Shimadzu 8900 using 40 scans and 4 cm⁻¹ resolution.

3. Results and discussion

As already mentioned, the design of the porous matrix is based on minor modifications of an already developed method [18]. Basically, we have replaced the porogen polystyrene beads with PMMA beads that can be also easily removed after the hardening of the macromolecular scaffold. Nevertheless we have used in this study smaller diameters (250 μ m) of the porogen compared with the previous reported approach (>650 μ m).

The hybrids were obtained as transparent solid porous scaffolds, with glassy appearance (Fig. 3). They present homogeneous spherical pores, separated through very thin polymer walls and perfectly interconnected through small round gaps. The dimension of the pores (250 μ m) was in good correspondence with the diameter of the porogen PMMA beads.

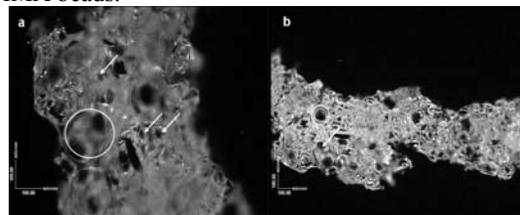


Fig. 3. Microscopic appearance of a porous polymer fragment: a) magnification 10x, b) magnification 5x; white arrows indicate for gaps between pores.

The high transparency of the materials made them very difficult to analyze by light microscopy. The porosity is the reason of multiple reflections of the light on the glassy walls of the polymer. Finally, the fragments appearing in Fig. 3 were observed as very thin slices cut from the original porous bulk material.

Since the porous scaffold is a hydroxylic polymer, the immobilization of the dendrimers was attempted after an intermediary chlorination of the hydroxyl groups on the surface of PHEMA. This step was performed in order to increase the reactivity of the polymeric surface towards the functional ending-groups of the dendrimers.

After the superficial chemical treatment, the hybrids suffered no changes in the porous morphology. We assume only nano-scaled roughness modification due to the presence of the dendritic globules on the surface, but light microscopy is not appropriate for this analysis. Further analysis will be performed.

Because the macromolecular hybrid is insoluble, the first qualitative evaluation of the dendrimer immobilization was realized by Fourier transform infrared spectroscopy (FTIR) - technique known to be suitable also for the investigation of the bone. Non-modified PHEMA was used as a control. This task has proven to be very difficult since the expected modifications occur only on the surface of the scaffolds.

Since PAMAMs are synthetic proteins based on a tree-like structure containing β -alanine units, for both ending-types dendrimers (amine and carboxyl) amide-characteristic peaks were anticipated. Specific amine and acid-specific peaks are also expected to appear compared with non-modified PHEMA.

Table 1 shows the assignment of the observed most representative peaks.

Fig. 4 presents the FTIR spectra of modified versus unmodified polymers.

The assignment of the recorded peaks was based on a direct comparison between control and modified-samples as well as on theoretical absorptions of different chemical groups. As seen in Fig. 4 and Table 1, the biggest discrepancies between the modified and control samples are represented, as expected, by the bands characteristic to amide groups. They can be considered and used as FTIR-“labels” for the detection of dendrimers.

The region from 1600 to 4000 cm^{-1} reveals the fundamental stretching vibration bands that can be assigned to specific diatomic pairs [21]. The intense absorption peak in the region 3300-3600 cm^{-1} common to all the recorded spectra, indicates the presence of OH from the PHEMA matrix. We assume that the intense and broad band observed at around 3400 cm^{-1} could be the result of the coupling of the intense -OH band with the weak overtone characteristic to carbonyl stretch at 1725 cm^{-1} . This weak intensity overtone is common for carbonyl-

containing compounds and occurs at twice the frequency of the fundamental vibration, 3450 cm^{-1} .

In the spectrum of amine-terminal PAMAM modified sample, in the region 3300-3600 cm^{-1} the shape of the registered band is different compared with the PHEMA and acid-modified sample. We assign this to the interference / overlapping of the OH stretching from the PHEMA matrix with the weaker NH stretching band at 3460 cm^{-1} corresponding to free amine groups in PAMAM. NH...O intermolecular interactions are not excluded and their contribution to the broadening of this band could be also assumed.

No other important informative peaks are present in the region 2800-3300 cm^{-1} , except the common C-H stretching and bending at around 2950 and 2880 cm^{-1} .

As previously mentioned, at 1725 cm^{-1} all the spectra show the presence of carbonyl from the PHEMA scaffold.

The region from 400 to 1600 cm^{-1} contains vibrations of mixed origin, many overtones and combination bands [21]. We have used this region to identify our FTIR-“labels” associated to dendrimer presence onto the surface of the scaffolds. Amide I and amide II appear around 1650 and 1558 cm^{-1} in both modified samples.

One may also notice that the peaks are more intense in the amine-containing scaffold compared with the carboxyl-containing polymer.

Table 1. Assignment of the main FTIR peaks.

Peak assignment	PHEMA	PHEMA-PAMAM amine ending	PHEMA-PAMAM carboxyl-ending
$\nu(\text{OH})^*$	3436 cm^{-1}	3400 cm^{-1}	3436 cm^{-1}
$\nu(\text{NH})$	-	3460 cm^{-1}	-
$\nu, \delta(\text{CH}_3) + \nu, \delta(\text{CH}_2)^*$	2950.9 + 2885 cm^{-1}	2962.80 + 2885 cm^{-1}	2952.8 + 2887.2 cm^{-1}
$\nu(\text{C=O})^*$	1728 cm^{-1}	1732 cm^{-1}	1732 cm^{-1}
$\nu(\text{C=O})$ Amide I	-	1651 cm^{-1}	1654 cm^{-1}
$\delta(\text{NH}) + \nu(\text{CN})$ Amide II	-	1556 cm^{-1}	1556 cm^{-1}
$\nu(\text{C-O})^*$	1000-1159 cm^{-1}	1000-1153 cm^{-1}	1000-1157 cm^{-1}
$\nu(\text{C-N})$	-	1080 cm^{-1}	-

*Bands assigned to both modified and unmodified polymers

This can be explained by the difference between the structures of amine- and acid-PAMAM molecules. In order to contain the same number of ending groups, the amine-PAMAM is full generation and the acid-PAMAM just half generation according to their synthesis. They are synthesized from ethylenediamine (EDA) core with Michael addition of methyl acrylate (MA) and amidation reactions of the resulting ester with large excess of EDA. Repetition of this sequence gives full generation $G=0,1,2,3,\dots$ (amine

terminal groups), while the interruption of this reactions sequence after the Michael addition leads to half generations $G=0.5, 1.5, 2.5, \dots$ (carboxy-terminal dendrimers) [17]. This is the main reason why the amine-ending dendrimer has more amide groups than the acid-one. This difference is clearly reflected on the intensity of the amide peaks in the FTIR spectra in Fig. 4.

The further analysis of the spectra shows the C-O stretching band for aliphatic alcohols (PHEMA) at $1000\text{--}1200\text{ cm}^{-1}$ in all the samples.

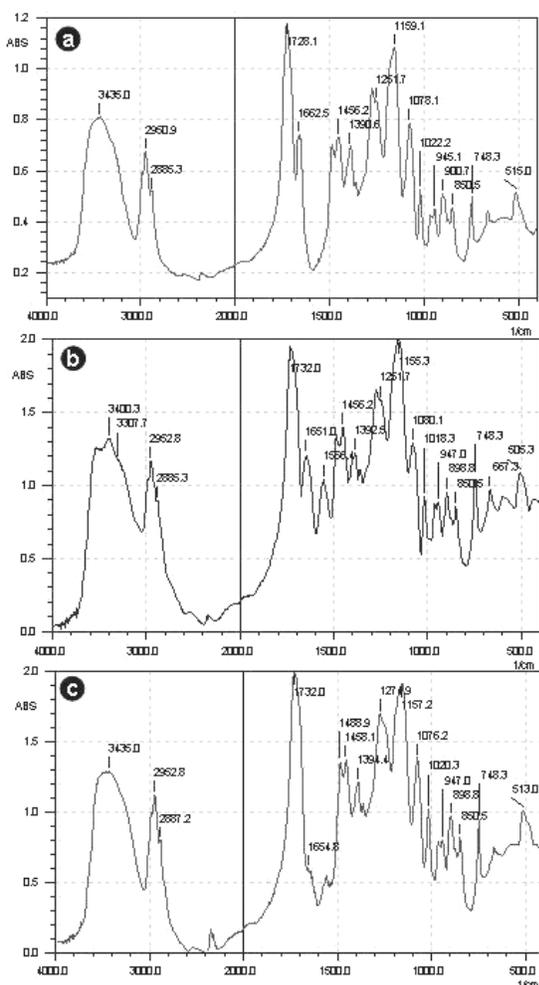


Fig. 4. FTIR spectra (absorbance) for a) PHEMA, b) PHEMA surface modified with amine-ending PAMAM, c) PHEMA surface modified with carboxyl - ending PAMAM.

A second “label”- like spectral difference could be noticed for the amine-modified samples at 1080 cm^{-1} , due to the interference of C-O stretching with the C-N stretches for amines (occurring at $1020\text{--}1200\text{ cm}^{-1}$ [21]).

This leads to a less visible separation between the bands at 1150 and 1080 cm^{-1} , when compared with the control PHEMA and with the acid-PAMAM modified scaffold.

Further assignment of the peaks $< 1000\text{ cm}^{-1}$ was not representative for our study.

Despite the surface-character of the modification, because PAMAM are large molecules with important number of amide and terminal groups, their presence on both-modified scaffolds is proved.

4. Conclusions

This work reports the design of hybrid materials polymer-dendrimer in a new approach of obtaining smart biomaterials for bone regeneration. The research efforts were aimed at the further application in orthopaedics, both through the mimic of trabecular bone architecture (by open macropores creation) as well as through the potential of the hybrid to in situ mineralize (template mineralization initiated by the terminal functional groups amine and carboxyl of dendritic components).

The porous structure was chosen both for the already explained biological aim and because it is extremely favorable to the chemical binding of the dendrimers since it increases the reactive surface of the macromolecular scaffolds.

The chemical approach is very challenging, the reactions occur at the interface solid-liquid, the quantitative extent of the modification is not very easy to establish.

Our first purpose was represented by the immobilization of the dendritic globules onto the matrix. The attempt to evaluate by FTIR the chemical modifications of the surface was successful and very promising, even if the reaction occurred only on the surface of the hybrid. Further research will focus on a deeper characterization of the modified surface in terms of homogeneity of the dendrimer layer, modification density and stability in physiological fluids. Several active surfaces will be created by using different generations and concentrations of dendrimers, with different functionalities, even mixed. New synthesis schemes will be also explored in order to find the best alternative. The evaluation of the *in vitro* HA-forming ability of the hybrids, for bone regeneration will be also performed.

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