THE EFFECT OF TITANIUM DIOXIDE ADDITION ON PHYSICAL AND BIOLOGICAL PROPERTIES OF Na_2O-B_2O_3-P_2O_5 AND CaO-Na_2O-P_2O_5 GLASSES

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Abstract

Two types of phosphate glasses 50Na_2O-20B_2O_3-30P_2O_5 (NBP) and 30CaO-20Na_2O-50P_2O_5 (CNP) with different content of TiO_2 (0, 3 and 5 mol%) have been prepared by melt-quenching process. TiO_2 was added to increase glass network stability. Physical properties of glasses were investigated by density measurements, differential scanning calorimetry and degradation in phosphate buffered saline (PBS). Biological performance of glasses in a direct contact with osteoblast-like MG-63 cells was analysed with the use of resazurin test and live-dead staining. The results show that TiO_2 addition increased density, glass transition temperature (T_g) and melting temperature (T_m) of both types of glasses. In the case of NBP glasses presence of TiO_2 resulted in their fast degradation in PBS and acidification of cell culture medium. As a consequence such glasses did not support cell adhesion and growth, but they can be considered for e.g. drug delivery systems. On the other hand addition of TiO_2 to CNP glasses resulted in enhanced cell adhesion and viability. Particularly positive results were found for CNP glass containing 5% TiO_2, so it can be a good candidate as a scaffold material for bone tissue engineering.

Keywords: *phosphate glasses, bioactive glasses, titanium dioxide, tissue engineering*

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Introduction

Treatment of bone defects caused by trauma or diseases is performed with the use of auto-, allo- and xenogeneic grafts, although their shortcomings are risk of disease transmission, limited amount, unpredictable resorption kinetics, etc. [1,2]. Therefore in the last two decades new biomaterials for replacement and regeneration of bone tissue defects have been intensively studied [3]. Particularly prospective alternatives are porous scaffolds, whose role is not only to support, but also to stimulate bone tissue ingrowth and remodelling. Fabrication methods of scaffolds include microsphere sintering, foam replication, electrospinning and rapid prototyping techniques [4]. Requirements for scaffolds and biomaterials from which they are made include:

- biocompatibility, no cytotoxic action,
- easy adhesion, proliferation and differentiation of cells on their surface,
- adequate degradation rate correlated with the tissue growth,
- bioactivity, i.e. the ability of formation of a direct bond between scaffold surface and bone tissue through low-crystalline hydroxyapatite layer,
- mechanical properties comparable to those of the replaced tissue,
- porous structure to facilitate tissue ingrowth,
- easy fabrication,
- susceptibility to sterilization [3,4,5].

Popular biomaterials for bone tissue engineering are bioactive glasses based on silica, especially 45S5 glass available under a trade name Bioglass®. However, despite its clinical application, for example for orthopaedic bone grafting (*NovaBone)* [6] these glasses have also some drawbacks, e.g.:

- prolonged biodegradation, which is difficult to control and causes problems with matching the degradation rate of the glass and the growth rate of new tissue,
- incomplete transition of glass into hydroxyapatite and presence of SiO_2 that may cause long-term side effects,
- difficult sintering process due to the viscous flow of the glass above its glass transition temperature (T_g) and the tendency to crystallize because of narrow window between T_g and crystallization temperature (T_c), which affects strength of the produced scaffold,
- high sintering temperature – addition of Na_2O and CaO decreases temperature, but it may simultaneously cause decrease in bioactivity, as a consequence of glass crystallization [3,7,8,9].

According to the above-mentioned drawbacks of the silica glasses, there is a rationale for the development of new bioactive glasses for tissue engineering. In comparison to the silica-based glasses, phosphate glasses have lower melting temperature, what makes fabrication of scaffolds easier. Moreover their biodegradation rate can be easily controlled by modifying the composition of glass, for example by the addition of TiO_2, and finally P_2O_5 is a component of hydroxyapatite, an integral part of bone extracellular matrix [8,10].

In this study we obtained and investigated phosphate glasses from two systems: Na_2O-B_2O_3-P_2O_5 (NBP) and CaO-Na_2O-P_2O_5 (CNP) as pure and modified with TiO_2. TiO_2 is known as a network modifier, which allows to control solubility of glasses and can improve cellular response [11]. Pure CNP glass and those glasses modified with TiO_2 were investigated in other studies [12]. According to our knowledge NBP glasses have not been examined so far as materials for bone tissue engineering.

Experimental

Glass synthesis

For glass fabrication we used conventional melt quenching process. NBP type glasses were prepared from Na_2CO_3 (99.99%), $NH_4H_2PO_4$ (pure) and H_3BO_3 (99.99%) in a 2.5:3:2 molar ratio as described before [13]. The synthesis of NBP glasses was carried out in the alumina mortar at 920°C. CNP type glasses were prepared from Na_2CO_3, $CaCO_3$ and P_2O_5 powders. The synthesis was two-step process including melting in the alumina mortar at 1075°C and then melting in the platinum crucible at 1050°C. The composition of obtained glasses is summarized in TABLE 1. FIG. 1 shows gross morphology of obtained glasses.

Density

We used pycnometer method to determine density of glasses. First, mass of the glass sample was measured, then mass of the pycnometer with water and mass of the pycnometer with water and sample were measured. Measurements were repeated three times for each sample. The density was calculated from the formula:

$$d_s = \frac{m_s \cdot d_w}{m_w + m_s - m_{ws}}$$

where:
d_s – density of the sample,
m_s – mass of the sample,
m_w – mass of the pycnometer with water,
m_{ws} – mass of the pycnometer with water and sample,
d_w – density of water.

Results are shown as average ± standard deviation.

Differential scanning calorimetry

Differential scanning calorimetry measurements were carried out on simultaneous thermal analyser (Netzsch STA 449F1Jupiter®). We used powder samples of about 30 mg. Heating rate was 10°C/min. Measurements were carried out in argon atmosphere. Glass transition temperature (T_g) was determined as characteristic inflection point on heating curve. Exothermic peak on heating curve was interpreted as crystallization of material; endothermic peak was connected with melting of material. Temperatures corresponding to the onsets of crystallization process and melting process were determined as crystallization temperature (T_c) and melting temperature (T_m), respectively.

Degradation

Degradation of glass samples was investigated in phosphate-buffered saline (PBS, Life Technologies). The composition of PBS was KH_2PO_4 (144 mg/L), NaCl (9000 mg/L), $Na_2HPO_4{\cdot}7H_2O$ (795 mg/L); pH was 7.4. We prepared set of three samples of each type of glass for several time points: 1 h, 3 h, 6 h, 12 h, 24 h, 3 days and 7 days for pure glasses and 3 h, 6 h, 12 h, 24 h, 3 days and 7 days for glasses modified with TiO_2. Masses of the samples were in the range of 30-50 mg. Each sample was placed in sterile polystyrene container with 10 mL of PBS. Degradation test was carried out at 37°C and pH of PBS was measured. At each time point samples were collected, dried and then weight of the samples after drying to a constant weight was measured.

Biological evaluation

Before *in vitro* tests glass plates (1 cm x 1 cm) were sterilised by immersion in 70% ethanol for 5 min followed by UV exposition for 20 min for both sites and placed in 12-well cell culture plates (EuroClone). MG-63 osteoblast-like cells (European Collection of Cell Cultures, Salisbury, UK) suspended in minimal essential medium (MEM, PAN™ BIOTECH, Germany) supplemented with 10% foetal bovine serum, 1% penicilin/streptomicin, 2 mM/L-glutamine (PAA, Austria) were seeded on each sample at initial density of $1x10^4$ cells/ml at 37°C under 5.0% CO_2 atmosphere and cultured for 24 h, 3 days and 7 days. Tissue culture polystyrene (TCPS, i.e. bottom of the well plates) was used as control. Cell viability was evaluated using In Vitro Toxicology Assay Kit, based on resazurin (Sigma Aldrich). 0.1 ml of rezasurin reagent was added to each well and the cells were incubated for 4 h at 37°C. Reduction of rezasurin was measured fluorescently (excitation wavelength 530 nm, emission wavelength 590 nm) (FLUOstar Omega, BMG labtech, Germany) and calculated according to the formula:

$$\%\ \text{Reduction of resazurine} = \frac{S^x - S^{control}}{S^{100\%\ reduced} - S^{control}} \cdot 100\%$$

where:
S^x – fluorescence of samples
$S^{control}$ – fluorescence of medium without cells
$S^{100\%\ reduced}$ – fluorescence of reagent reduced in 100% (reagent with medium was placed in autoclave for 15 min at 121°C).

Results were expressed as average ± standard deviation (SD) from three independent samples performed in triplicate. Statistical significance was evaluated according to the unpaired t-test; $p<0.05$ was considered significant.

TABLE 1. Composition of obtained glasses.

Type of glass	P_2O_5 % mol	Na_2O % mol	B_2O_3 % mol	CaO % mol	TiO_2 % mol
NBP	30	50	20	-	0
CNP	50	20	-	30	0
NBP: 3% TiO_2	30	47	20	-	3
NBP: 5% TiO_2	30	45	20	-	5
CNP: 3% TiO_2	50	17	-	30	3
CNP: 5% TiO_2	50	15	-	30	5

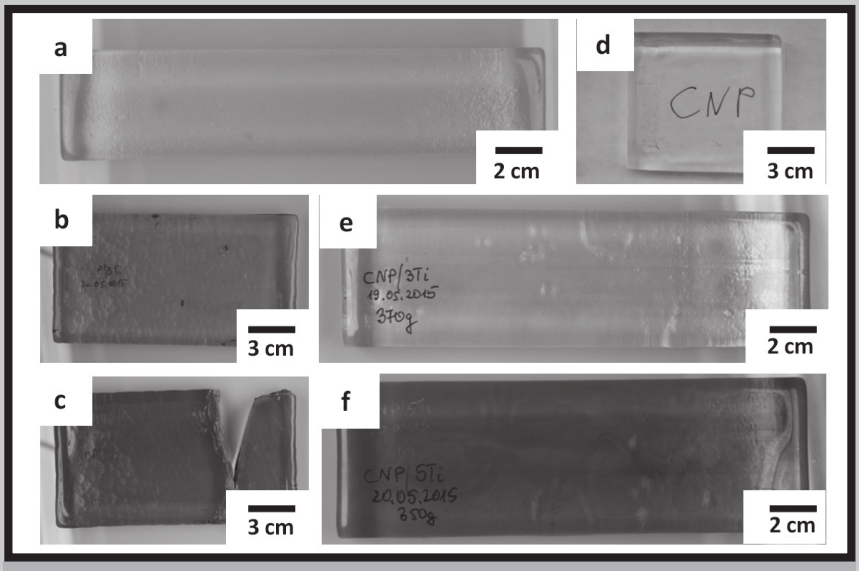

FIG. 1. Gross morphology of glass samples: a) NBP; b) NBP 3% mol TiO_2; c) NBP 5% mol; d) CNP; e) CNP 3% mol TiO_2; f) CNP 5% mol TiO_2.

To visualize cell attachment and distribution on the samples, the cell cultures were observed using fluorescence microscopy (Zeiss Axiovert, Germany). A *live-dead* staining was performed to evaluate cell viability. Samples were rinsed with PBS (PAN™ BIOTECH, Germany) and the supernatant was replaced by 1 ml PBS solution containing 2 µl (1 mg/ml) calcein AM and 2 µl (1 mg/ml) propidium iodide (Sigma-Aldrich).

Results and discussion

Density

TABLE 2 shows results of density measurements. Density of pure NBP glass is slightly lower than density of pure CNP glass. TiO_2 addition increases density of both types of glass. For NBP glasses densities vary from 2.548 ±0.016 g/cm^3 for pure NBP glass to 2.601 ±0.017 g/cm^3 for NBP glass with 5 mol% TiO_2. Addition of 5 mol% TiO_2 in the case of CNP glasses changes density from 2.588 ±0.004 g/cm^3 for pure CNP glass to 2.621 ±0.007 g/cm^3. It has been reported that TiO_2 addition changes the structure of sodium borophosphate and soda-lime phosphate glasses by formation of P-O-Ti bond instead of P-O-P and P-O-B bonds [12,14,15]. Presence of titanium ions leads to increase in network cross-links and results in denser packing of the structure, hence we observed increase in density for glasses containing TiO_2 [14].

TABLE 2. Density of glasses.

Type of glass	Density [g/cm^3]	Type of glass	Density [g/cm^3]
NBP	2.548 ±0.016	CNP	2.588 ±0.004
NBP 3 mol% TiO_2	2.586 ±0.010	CNP 3 mol% TiO_2	2.595 ±0.004
NBP 5 mol% TiO_2	2.601 ±0.017	CNP 5 mol% TiO_2	2.621 ±0.007

Differential scanning calorimetry

TABLE 3 shows characteristic temperatures of glasses. Glass transition temperature (T_g) of NBP is 432.5°C and increases to 436.9°C for NBP glass containing 3 mol% TiO_2 and to 447.7°C in the case of 5 mol% TiO_2 addition. Similarly, TiO_2 addition causes increase in melting temperature (T_m). In contrast, addition of 3 mol% TiO_2 decreases crystallization temperature (T_c) from 596.7°C to 579°C, but addition of 5 mol% TiO_2 slightly increases T_c to 603.2°C. In comparison to NBP glass, CNP glass has slightly lower glass transition temperature of 412.3°C. TiO_2 addition to CNP glasses causes increase of T_g from 412.3°C to 437.4°C, and T_c from 536.7°C to 602.4°C and T_m from 682.5°C to 749°C. Increase in thermal stability as well as increase in densities for TiO_2 containing glasses is a result of modification of glass network by TiO_2 which is due to depolymerisation of borate and phosphate chains and formation of P-O-Ti bonds [14,16]. FIG. 2 shows DSC curves for NBP type and CNP type glasses. Presence of multiple endothermic and exothermic peaks is connected with transformations of different glass phases. For unmodified CNP glass we observe two crystallization peaks and three melting peaks. Two crystallization peaks and two melting peaks appear for CNP glass containing 3 mol% TiO_2. For CNP glass with 5 mol% TiO_2 we observe two crystallization peaks and one melting peak.

TABLE 3. Characteristic temperatures of glasses.

Type of glass	Glass transition temperature T_g [°C]	Crystallization onset temperature T_c [°C]	Melting onset temperature T_m [°C]
NBP	432.5	596.7	743.9
NBP 3 mol% TiO_2	436.9	579.0	759.2
NBP 5 mol% TiO_2	447.7	603.2	760.9
CNP	412.3	536.7	682.5
CNP 3 mol% TiO_2	420.5	581.2	738.7
CNP 5 mol% TiO_2	437.4	602.4	749.0

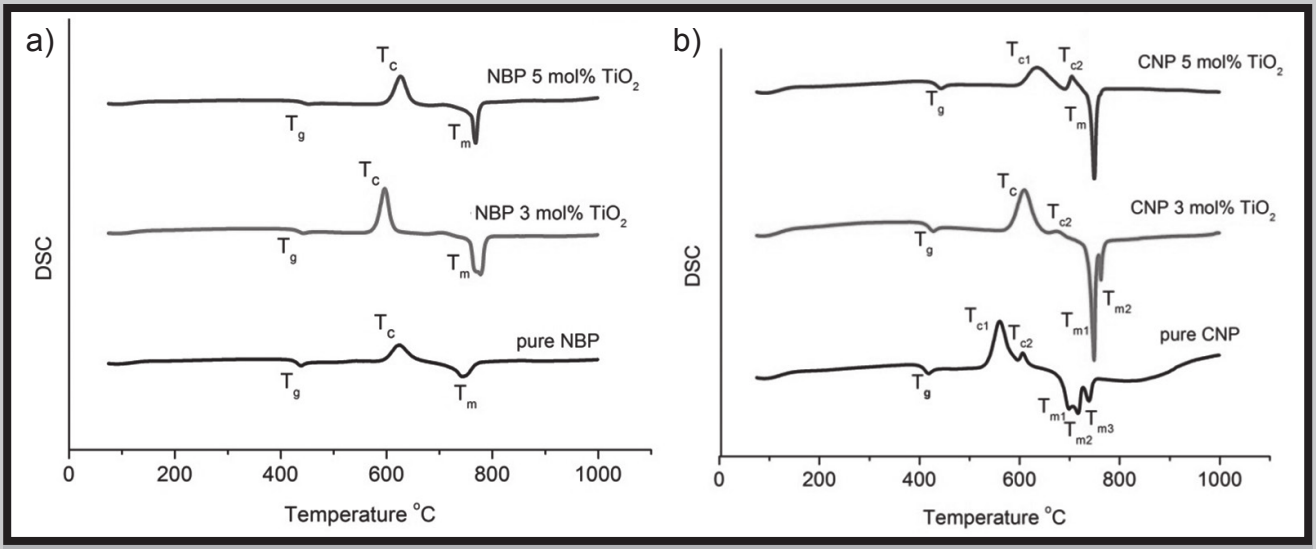

FIG. 2. DCS curves of: a) NBP type glasses; b) CNP type glasses.

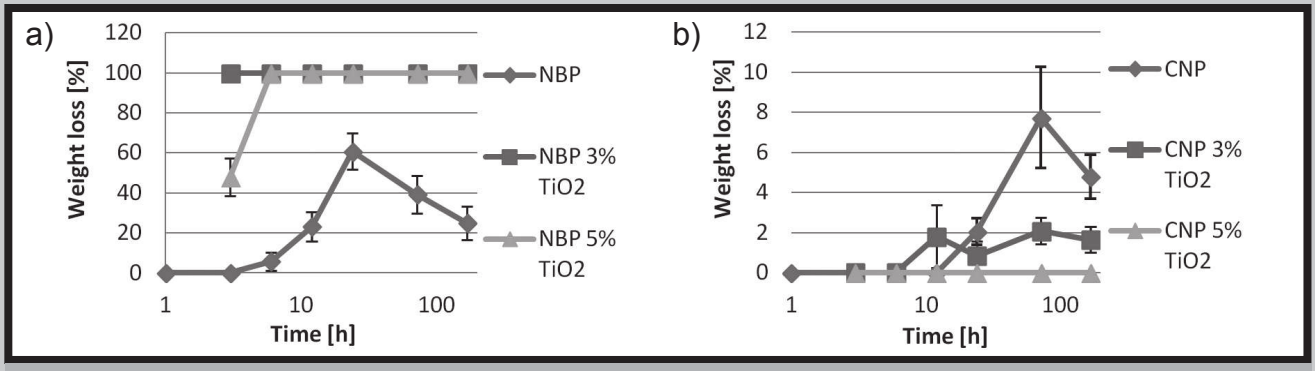

FIG. 3. Weight loss of glass samples as a function of time: a) NBP type glasses; b) CNP type glasses.

Degradation

FIG. 3 shows weight loss results of NBP (FIG. 3a) and CNP (FIG. 3b) type glasses as a function of incubation in PBS. Weight loss of NBP glass after 24 h is about 61 ±9%, but it decreases within the following days. Interestingly, after longer incubation time in PBS weight loss of NBP glass decreases. It can be connected with precipitation of new mineral phase on the glass surface [18]. Addition of TiO_2 as a network modifier does not improve stability of NBP glass (FIG. 3a). On the contrary, it results in very rapid glass dissolution: NBP 3% TiO_2 dissolves faster than that containing 5% TiO_2. CNP glass is much more stable: it starts to dissolve after 10 h and after 3 days its weight loss is about 7.7 ±2.5% (FIG. 3b). Addition of TiO_2 improves stability of CNP glasses. After 7 days weight loss of CNP glass with addition of 3 mol% TiO_2 is only 1.6 ±0.6%. Addition of 5 mol% TiO_2 causes that CNP glass is very stable and even after 7 days weight loss is not observed.

FIG. 4 shows picture of cell culture wells with cell culture medium (MEM) after 5 min from introduction of analysed glass samples. It is apparent that CNP glasses do not cause change in pH, which is 7.2, i.e. the same as on control TCPS. NBP glass causes acidification of cell culture medium: pH drops to 6.8 for NBP, and it drops again when the NBP structure contains TiO_2: 3%TiO_2 results in pH 6.0 and 5%TiO_2 results in pH 6.2.

Despite of increase in T_g temperature for TiO_2-containing NBP glasses, which could be connected with higher stability of glass structure, results of pH of cell culture medium and also weight loss measurements show that TiO_2 does not cause reinforcement of the NBP glass network. Hence, we observe pH decrease for glasses with TiO_2 addition, as shown by others [19].

The results of pH of PBS as a function of incubation of analysed glasses for 7 days are shown in FIG. 5. Incubation of NBP glass in PBS results in a small increase of pH from 7.2 to 7.5 after 1 h and after 7 days the pH of PBS increases to 8.4 (FIG. 5a). NBP glass containing TiO_2 causes decrease of pH to 7.02 ±0.05 for NBP with 3 mol% TiO_2 and 6.97 ±0.05 for NBP with 5 mol% TiO_2 after 7 days (FIG. 5a). The differences in the pH of cell culture medium and PBS can result from the fact that these two media have different chemical compositions. Moreover the ratio of glass mass to medium volume was higher for cell culture medium than for degradation test in PBS. Incubation of CNP glasses (FIG. 5b) in PBS causes slight increase of pH from 7.2 to 7.35-7.55, which is coherent with the results observed for samples incubated in cell culture medium (FIG. 4).

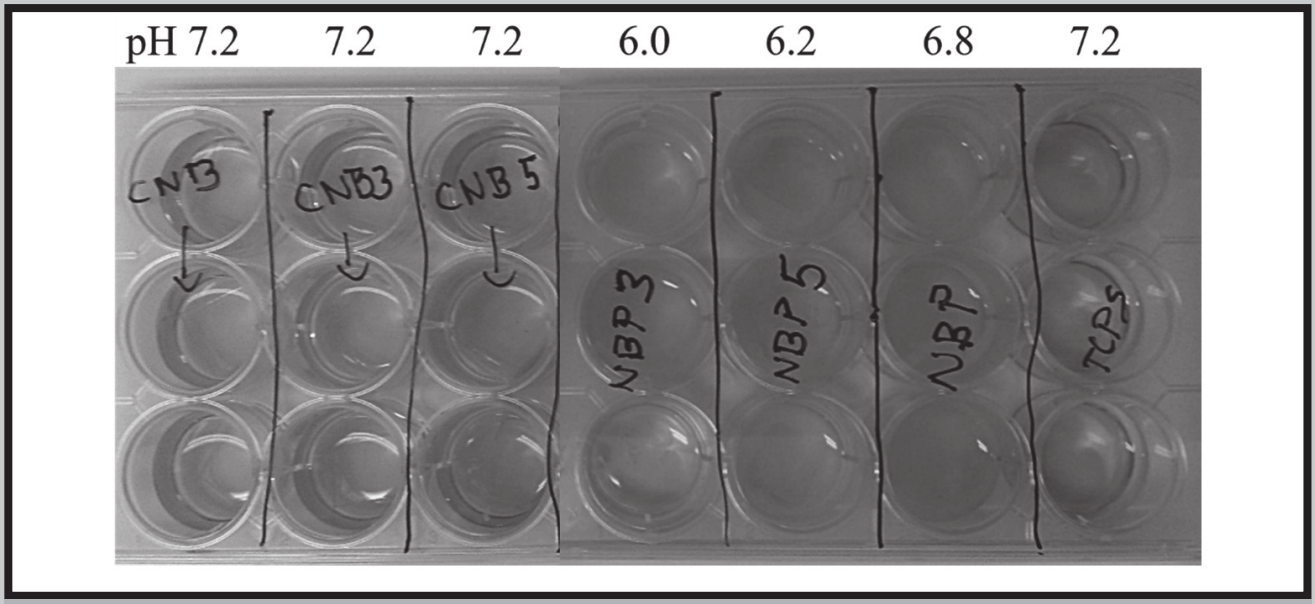

FIG. 4. Picture of cell culture wells containing analysed glasses with cell culture medium (MEM) supplemented with pH indicator phenol red after 5 min from introduction of analysed glass samples. Change in colour indicates pH of medium: CNP – pH 7.2, CNP 3%TiO_2 – pH 7.2, CNP 5%TiO_2 – pH 7.2, NBP – pH 6.8, NBP 3%TiO_2 – pH 6.0, NBP 5%TiO_2 – pH 6.2, and tissue culture polystyrene TCPS – pH 7.2.

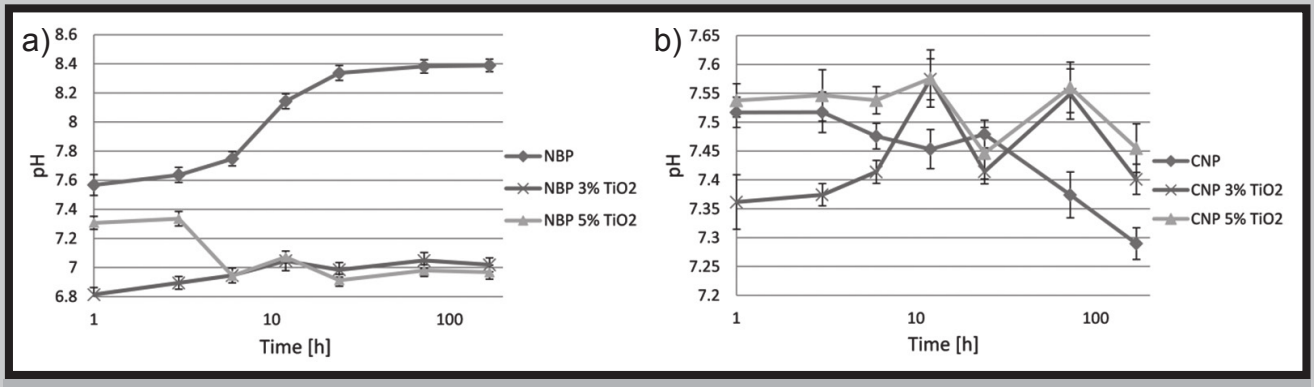

FIG. 5. pH of PBS in contact with glass samples as a function of time: a) NBP type glasses; b) CNP type glasses.

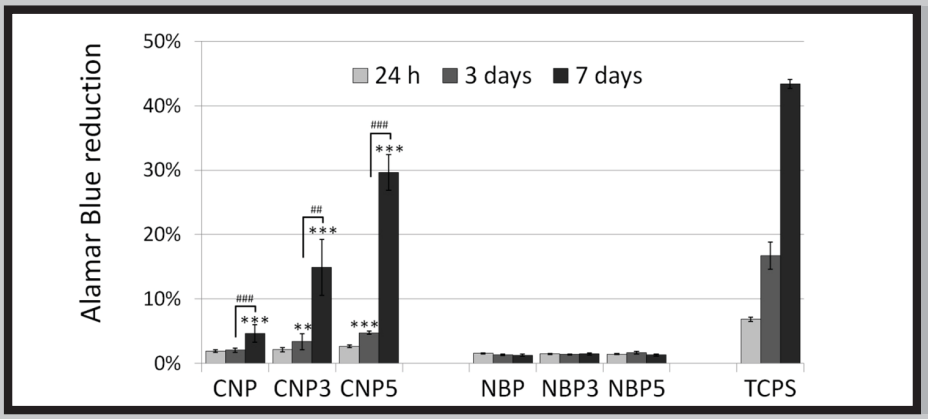

FIG. 6. Viability of MG-63 cells measured by resazurin reduction test. Statistic significant for TCPS is p<0.001 for all samples (not marked in the figure). Asterisks indicate significant differences (p<0.01 and p***<0.001) compared to control CNP and hashtags indicate significant differences ($p^{\#\#}$<0.01 and $p^{\#\#\#}$<0.001) between day 3 and day 7.**

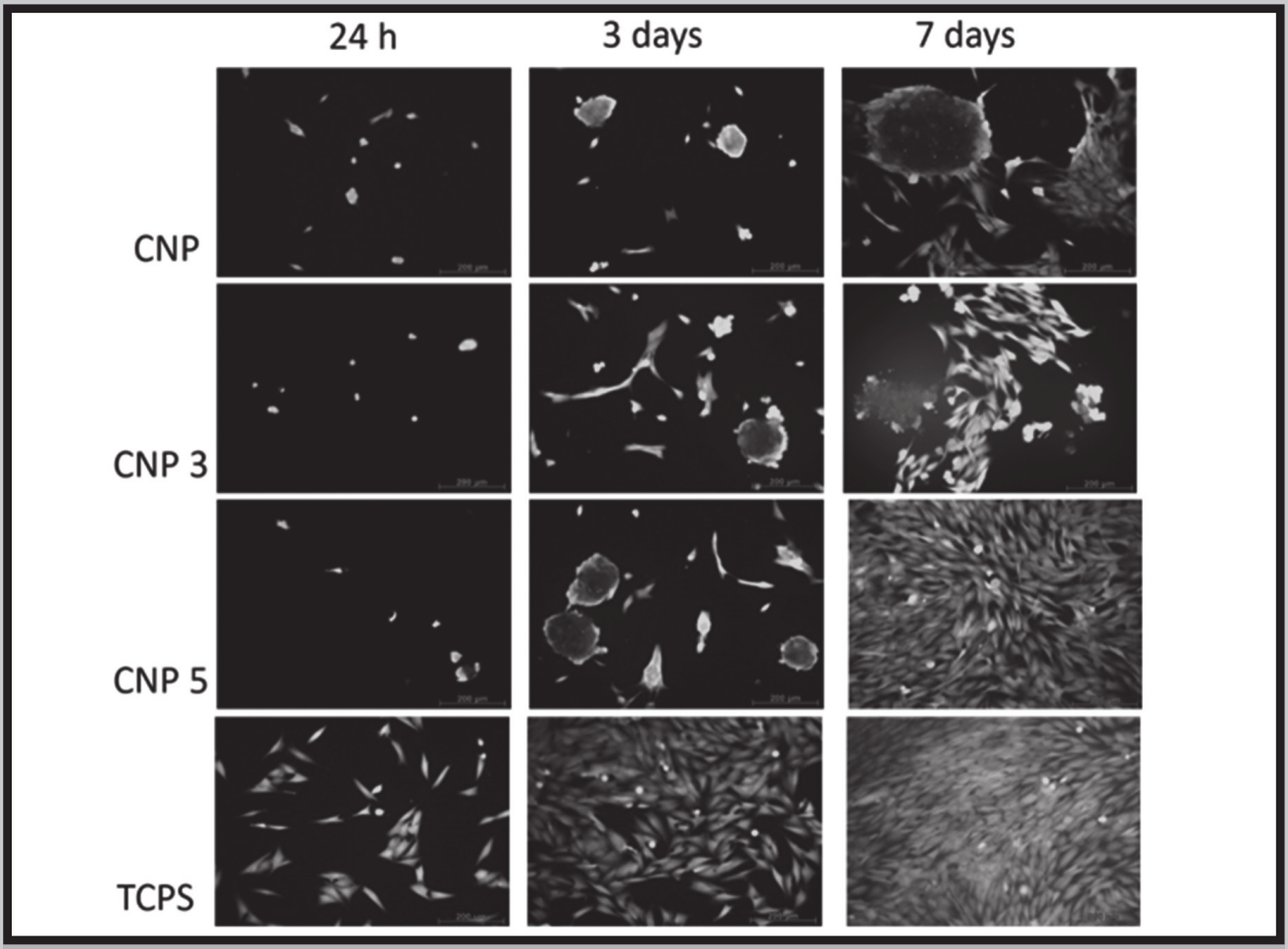

FIG. 7. Morphology and distribution of MG-63 cells stained live/dead on different glasses and control TCPS after 24 h, 3 days and 7 days. Scale bar 100 µm on all pictures.

Biological performance

Cell viability test (FIG. 6) shows that metabolic activity of MG-63 cells for CNP-type samples was reduced as compared to TCPS. The cells showed the tendency to increase their viability with culture time on CNP-type samples with increased concentration of TiO_2. After 3 and 7 days on CNP3 and CNP5 cell viability was significantly higher than on CNP. CNP5 exhibited the highest viability of cells as compared to CNP and CNP3. On NBP, NBP3 and NBP5 viability of cells was very low. It was due to the fact that the glass samples were dissolved in culture medium and pH of the medium became too acidic for cells to survive (FIG. 4).

FIG. 7 shows morphology of cells on the surface of the glasses stained in green (live cells) and red (dead cells). On CNP-type samples the number of adheared cells was smaller than on TCPS; the cells were round and poorly spread. After 3 days the cells on CNPs grew in agglomerates and some red dead cells were visible, especially inside the agglomerates. After 7 days on CNP5 the cells were homogenously distributed and formed monolayer (similar to that on TCPS), while on CNP and CNP3 big agglomerates containing both live (green) and dead cells (red) were visible. The number of cells at NBP-type glasses was very low and the observed cells were stained in red, i.e. they were dead (data not shown).

In vitro tests show that CNP-type glasses support adhesion and proliferation of bone cells but NBP-type glasses do not, which is connected with their too rapid degradation in aqueous environment.

Conclusion

We investigated physical and biological properties of glasses from two systems: Na_2O-B_2O_3-P_2O_5 (NBP) and CaO-Na_2O-P_2O_5 (CNP) as pure and doped with 3 or 5 mol% TiO_2. Increasing content of TiO_2 caused changing in density, thermal stability and degradation behaviour of glasses. In the case of NBP glass we observed increase in density, glass transitions temperature and melting temperature. Addition of 3 mol% TiO_2 did not increase crystallization temperature. NBP glasses were found to dissolve in aqueous environment and as a result they did not support growth of bone cells.

TiO_2 addition to CNP glasses caused increase in their density, glass transition temperature, crystallization temperature and melting temperature. Compared to NBP glass, increase in these characteristic temperatures was much more significant for CNP glass. This suggests that TiO_2 addition can change the structure of NBP and CNP glasses in a different way, hence we observed various influence of TiO_2 on their physical properties. CNP glasses supported adhesion, growth, proliferation and viability of osteoblast-like cells. The best properties as regards biological performance had CNP5 glass, although adhesion of cells was impeded at shorter time points (24 h, 3 days). Interestingly after 7 days the cells had similar morphology as on control TCPS, however their viability was still significantly lower.

To sum up NBP-type glasses are not suitable support for cell culturing, because they dissolve in cell culture medium and acidify it, provoking cell death, but they can be considered as drug delivery systems. On the other hand CNP-type glasses (especially those containing 5% TiO_2) can be used as a substrate for bone tissue engineering scaffolds. However further studies are needed to provide more clinically relevant evidences in this matter.

Acknowledgements

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