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Title: Novel whey protein isolate-based highly porous scaffolds modified with therapeutic ion-releasing bioactive glasses

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Keywords: gas foaming; bone tissue engineering; waste material; micro-computed tomography

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Abstract: In this work, for the first time, a material derived from food industry waste - whey protein isolate - and a material commonly used in bone regeneration - bioactive glasses - were combined to obtain novel composite biomaterials with potential applications in bone tissue engineering (BTE). Additionally, to obtain pro-angiogenic properties, sol-gel-derived BGs doped with Cu^{2+} and Co^{2+} ions were used. Using a simple gas foaming method, ready-to-use (sterile), bioactive scaffolds with high porosity (above 70%), fully connected pore networks, and pore size suitable for BTE applications (80-350 µm) were obtained. Furthermore, scaffolds showed additional functionalities - calcium phosphate-forming ability and gradual release of therapeutic ions. Porous WPI/BG composites showed great potential for use as novel bone substitutes.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

20th November 2019

Dear Dr. Y.F. Zheng,

Thank you for giving us the chance to submit a revision.

We are pleased to submit revised manuscript entitled *Novel whey protein isolate-based highly porous scaffolds modified with therapeutic ion-releasing bioactive glasses*.

The manuscript has been improved according to the Reviewers' suggestions. Red font indicates where changes have been made. We really hope that this manuscript will be acceptable for publication in ***Materials Letters***.

Many thanks for your attention and we look forward to hearing from you.

Yours sincerely

Michal Dziadek (corresponding author) and Co-authors

20th November 2019

We were grateful to receive all the valuable comments from the Reviewers. The manuscript has been improved according to the Reviewers' suggestions. Red font indicates where changes have been made. We really hope that this manuscript will be acceptable for publication in ***Materials Letters***.

Below you will find our detailed answers to the Reviewers' comments and suggestions.

Yours sincerely

Michal Dziadek (corresponding author)

Reviewer 1:

1. This paper need to work on results and discussion.

Results and discussion section have been revised. However, in view of limited total number of words in manuscript, according to journal requirements, the possibility to extend the Results and discussion section was limited.

2. The figure 1 is not clear.

Figure 1 has been modified to improve its readability. The sentence in Materials and methods section was added to clarify fabrication process of the materials.

Reviewer 2:

1. Authors should examine some of the minimal biological properties of the scaffolds, such as degradation, apatite formation and cell viability.

Calcium-phosphate formation (*in vitro* bioactive properties) on the surfaces of composite materials after incubation in SBF was previously confirmed by FTIR (the presence of characteristic bands at 558, 600, and 1014 cm^{-1}) and ICP-OES (decrease in concentrations of calcium and phosphate ions in SBF during incubation) (Figures 3A-3C). Additionally, SEM/EDX analyses were added (Figure 3D) and commented in the Results and Discussion section. As there is limited data on processing of WPI into biomaterials in the form suitable for tissue engineering (TE) applications, the main objective of this study was to demonstrate the possibility of fabricating highly porous scaffolds based on WPI using a simple foaming method and to investigate their microstructure (extremely important from the point of view of potential application in TE). We agree with the Reviewer that additional tests (*in vitro* degradation and cell studies) are essential, as we also emphasised in conclusions. However, in view of limited total number of words in manuscript, according to journal requirements, it is hard to enriched manuscript in additional data and discussed its thoroughly. These studies are ongoing and they will be the subject of further publication. It should be pointed out that our previous results indicate that both WPI (DOI: 10.1002/jbm.a.36754) and Cu and Co-doped glasses (DOI: 10.1016/j.matlet.2017.12.075) are cytocompatible, therefore we expected that porous WPI-based materials also show cytocompatibility. Therefore, more advanced research (e.g. osteogenic and angiogenic potential) should, in particular, be examined. We acknowledge the importance of the reviewer 's recommendation and have added a sentence to the end of the discussion.

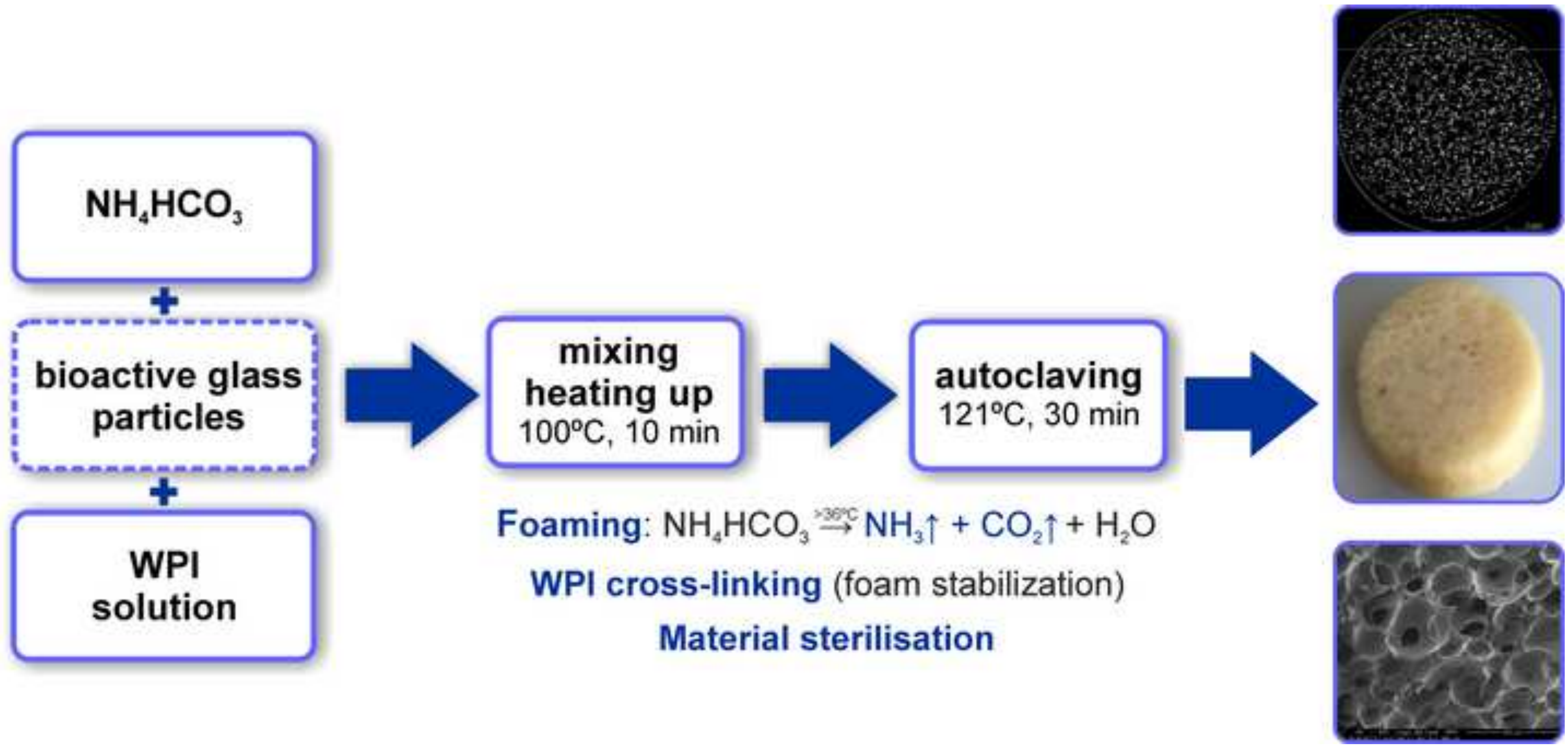
2. Pore connectivity should also be provided.

The parameters determined based on μCT (open porosity and total porosity), allowed us to conclude that that manufacturing process ensures nearly 100% interconnectivity of the pores. We have mentioned this in the text. Thank you for the suggestion.

3. Some recent key references on bone repair materials and scaffolds should be cited to feedback the research trend in this field. - de Grado, Gabriel Fernandez; Keller, Laetitia; Idoux-Gillet, Ysia. Bone substitutes: a review of their characteristics, clinical use, and perspectives for large bone defects management. J. Tissue Eng. 2018; 9: 2041731418776819; Winkler et al., A review of biomaterials in bone defect healing, remaining shortcomings and future opportunities for bone tissue engineering, Bone Joint Res., 2018, 7(3): 232-243.

Discussion section have been revised and the references were cited in manuscript.

Graphical Abstract



***Highlights (for review)**

Highly porous scaffolds based on WPI and Cu- and Co-doped bioactive glasses were developed

Simple gas foaming method allowed for obtaining ready-to-use (sterile) materials

Scaffolds showed porosity suitable for bone tissue engineering applications

Scaffolds exhibited bioactivity and the ability to gradually release proangiogenic ions

*Manuscript

Novel whey protein isolate-based highly porous scaffolds modified with therapeutic ion-releasing bioactive glasses

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ABSTRACT

In this work, for the first time, a material derived from food industry waste – whey protein isolate - and a material commonly used in bone regeneration – bioactive glasses - were combined to obtain novel composite biomaterials with potential applications in bone tissue engineering (BTE). Additionally, to obtain pro-angiogenic properties, sol-gel-derived BGs doped with Cu^{2+} and Co^{2+} ions were used. Using a simple gas foaming method, ready-to-use (sterile), bioactive scaffolds with high porosity (above 70%), fully connected pore networks, and pore size suitable for BTE applications (80–350 μm) were obtained. Furthermore, scaffolds showed additional functionalities - calcium phosphate-forming ability and gradual release of therapeutic ions. Porous WPI/BG composites showed great potential for use as novel bone substitutes.

KEYWORDS: gas foaming; bone tissue engineering; waste material; micro-computed tomography

1. INTRODUCTION

The search for new or alternative materials for use in tissue engineering (TE) is one of the main challenges. Recently, there has been a great deal of interest in waste raw materials as they are environmentally friendly, inexpensive and widely available [1]. One of these materials is whey protein isolate (WPI), a by-product of cheese manufacturing. Heat treatment of an aqueous solution of WPI above 60 °C results in unfolding of the proteins followed by the formation of interprotein bonds, leading to the formation of a three-dimensional hydrogel network [2,3]. Its ability to form a hydrogel without the use of chemical cross-linking agents and biodegradability makes WPI attractive for use in biomedical applications. Previous studies have indicated that WPI dissolved in cell culture medium stimulates osteogenic differentiation of bone-forming cells [4,5], indicating its potential use in bone tissue regeneration. However, there is still limited data on biological properties of the WPI-based biomaterials, especially in a form suitable for TE applications (scaffolds, membranes) [6].

A group of widely studied biomaterials for BTE includes bioactive glasses (BGs). Attractiveness of BGs lies in their ability to bond to living bone (bioactivity) [7]. Furthermore, their ionic dissolution products have been shown to stimulate bone formation via activating osteogenic genes (osteoinductivity) [8]. Because of their ability to incorporate a large variety of elements and their controllable dissolution properties in physiological fluids, BGs can be used as effective carriers for inorganic therapeutic ions. This brings new opportunities to enhance osteogenic activity of BGs (Mg^{2+}, Sr^{2+}, Zn^{2+}) and/or to impart them new biological functionalities, e.g. antibacterial (Zn^{2+}, Cu^{2+}, Ag^{+}) and angiogenic properties (Cu^{2+}, Co^{2+}) [9]. The latter has recently attracted particular attention because angiogenesis, besides osteogenesis, is essential for complete bone tissue regeneration. One more advantage of BGs is their application as modifiers in polymer composite materials, to combine the aforementioned advantages of BGs and advantages of polymers, including tunable biodegradability and ease of processing into various forms [10].

In this work, for the first time, a material derived from food industry waste – WPI - and a material commonly used in bone regeneration – BGs - were combined to obtain novel composite biomaterials with potential applications in BTE. Additionally, to obtain pro-angiogenic properties, BGs doped with Cu^{2+} and Co^{2+} ions were used. Simple method based on the gas foaming was applied to produce highly porous WPI/BG scaffolds.

2. MATERIALS AND METHODS

WPI was purchased from BiPro, Davisco Foods International Inc., USA. The copper and cobalt-doped bioactive glasses (A2Cu5 and A2Co5, respectively) from $40SiO_2$–$49CaO$–$5MeO$–$6P_2O_5$ (mol.%) system (where Me – Cu, Co) were synthetized using sol-gel method as was described previously [11].The glasses were milled and sieved to obtain the powder with particle size <45µm. The scaffolds were produced using ammonium hydrogen carbonate (NH_4HCO_3, POCh, Poland) as foaming agent, according to the scheme shown in Fig. 1. Briefly, NH_4HCO_3 was mixed with bioactive glass particles and aqueous WPI solution in 2-ml microcentrifuge tubes using a vortex mixer. Afterwards the mixture in tightly closed tubes was mixed and heated to 100°C for 10 minutes using a thermomixer. Tubes were then opened and autoclaved at 121 °C for 30 minutes. After autoclaving, tubes were closed under sterile conditions and stored in the fridge until further analysis. Reference materials (WPI), unmodified with BGs, were also produced by mixing NH_4HCO_3 with WPI solution.

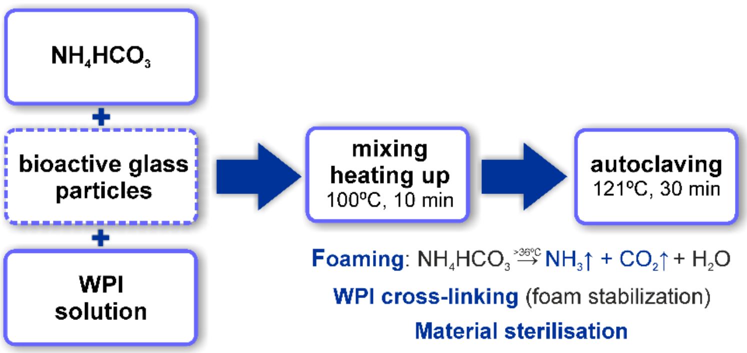

Figure 1. Scheme of the preparation process of highly porous WPI-based scaffolds.

The samples were analyzed with SEM (Nova NanoSEM 200 FEI Europe Company, Netherlands) after lyophilization and coating with a thin conductive carbon layer. µCT scans were recorded using a SkyScan 1272 high-resolution X-Ray microtomograph (Bruker MicroCT, Belgium). CT Analyser (Version 1.17.7.2) software was used for quantitative data regarding the samples' porosity. Open porosity was determined with mercury intrusion porosimetry (PoreMaster60, Quantachrome Instruments, UK). *In vitro* bioactivity was evaluated by incubation of materials in simulated body fluid (SBF) for 3, 7 and 14 days. The samples were analyzed using ATR-FTIR (Bruker VERTEX 70V spectrometer, USA) and SEM/EDX methods. The changes in Ca, P, Si, Cu, and Co concentration in the SBF were monitored using an ICP-OES technique (Plasm 40, Perkin Elmer, USA). For all sample groups, n = 3.

3. RESULTS AND DISCUSSION

Highly porous WPI-based composite scaffolds were fabricated for the first time by applying simple gas foaming method. As shown in Fig. 1, during heating and autoclaving, three processes took place. Thermal decomposition of NH_4HCO_3 with the release of gaseous products (NH_3 and CO_2) ensured foaming of the WPI solution. Temperature above 60 °C allowed for WPI cross-linking, which at the same time stabilizes the foam. Finally, the manufacturing process also ensured the sterilization of the material – there is no need to apply an additional step. This is an important advantage because it allows to meet one of the basic requirements for biomaterials - ease and efficiency of sterilization without changing the material properties [12].

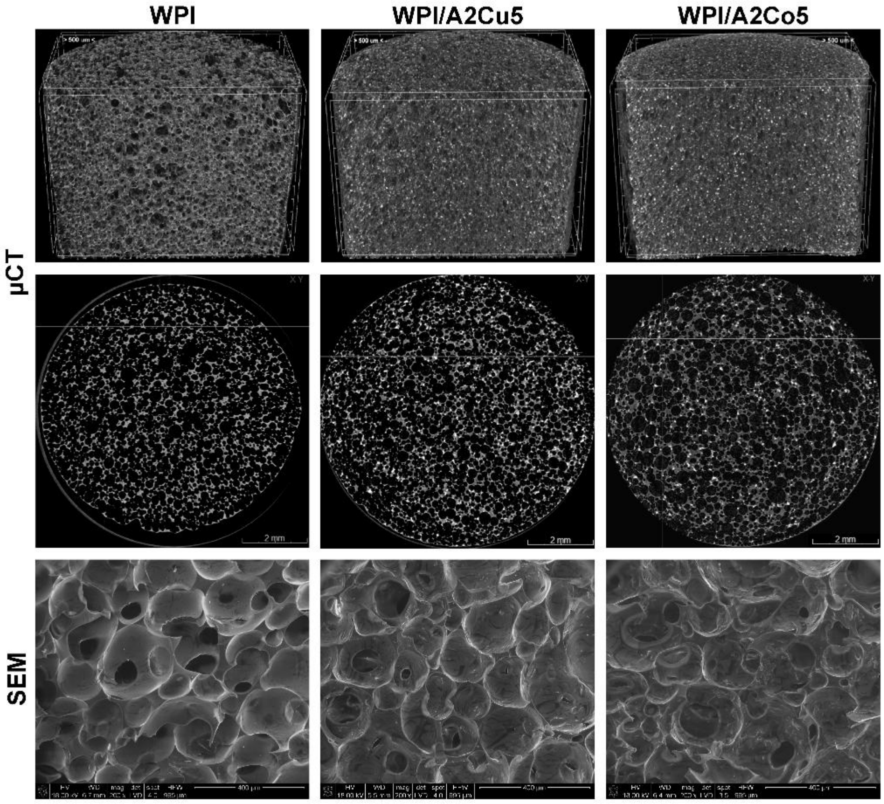

Figure 2. μCT-based visualizations and SEM images of the WPI-based scaffolds.

SEM analysis showed that scaffolds have highly interconnected, round-shaped pores with 80–350 μm diameter (Fig. 2). Furthermore, scaffolds revealed high porosity above 70% (Table 1). Importantly, the same values of open porosity and total porosity, determined based on μCT, indicate that manufacturing process ensures nearly 100% interconnectivity of the pores. Such pore size, porosity and interconnectivity is considered ideal for bone regeneration and vascularization, ensuring cell migration, extracellular matrix secretion, ingrowth of blood vessels, as well as transport of oxygen, nutrient and wastes [13]. The presence of BG particles in WPI matrix did not influence significantly above mentioned porosity parameters. Pore walls of the WPI/BG materials became rougher, which may additionally promote cell adhesion [14].µCT analysis showed homogenous distribution of BG particles in WPI matrix.

Table 1. Material porosity determined based on microcomputed tomography (µCT) and mercury intrusion porosimetry (MIP).

Material	Open porosity (%) µCT	Total porosity (%) µCT	Open porosity (%) MIP
WPI	78.2 ± 2.3	78.2 ± 1.5	82.5 ± 2.0
WPI/A2Cu5	77.8 ± 1.8	77.8 ± 2.7	78.2 ± 1.3
WPI/A2Co5	74.9 ± 2.1	74.9 ± 2.6	70.6 ± 2.9

In the case of WPI scaffold, concentration of calcium and phosphate ions in SBF did not change significantly over incubation time (Fig. 3A). For WPI/BG composites, Ca^{2+} content increased during first 3 days and then gradually decreased until the end of incubation. Ca^{2+} concentration in medium was influenced by ion release from the glass particles and ion uptake resulting from the formation of calcium phosphate (CaP) layer [15]. These results corresponded to the consumption of phosphate ions, confirming CaP layer formation. Furthermore, the release of Si^{4+} and pro-angiogenic ions (Cu^{2+}, Co^{2+}) from the composite structure was noticed (Fig. 3B). In the case of WPI/A2Cu5, reduction of the concentration of Si^{4+} and Cu^{2+} ions after 7 days may result from the incorporation of them into bioactive layer. FTIR spectrum of WPI hydrogel did not show any significant changes that could indicate the formation of CaP layer after incubation in SBF (Fig. 3C). However, FTIR spectra of both composite materials incubated for 14 days consist of new bands at 558 and 600 cm^{-1} (O–P–O bending mode), as well as 1014 cm^{-1} with a shoulder at 1115 cm^{-1} (P–O stretching mode) characteristic for calcium phosphates [16]. The presence of the layers rich in calcium and phosphorus on the surfaces of WPI/BG composites after 14-day incubation in SBF was also confirmed with SEM/EDX (Fig. 3D). Both FTIR and SEM/EDX analyses indicate potential bioactive properties of WPI/BG scaffolds.

The lack of vascularization of bone substitutes is identified as a key limitation for successful bone regeneration, especially in case of large defects. Besides adequate porosity, another approaches to promote vascularization is the use of angiogenic factors such as vascular endothelial growth factor (VEGF) or encapsulating angiogenic cells [17,18]. However, this strategy suffers from a number of disadvantages, such us high instability and sensitivity, short half-life, ability to provoke an undesirable immune response, the low viability of the transplanted cells, and finally high cost [19]. Therefore, controlled delivery of inorganic ions, in particular Cu^{2+} and Co^{2+} ions, which are capable to mimic hypoxia in normoxic conditions by artificially stabilizing HIF-1α (hypoxia-inducible factor 1α) expression, has recently been widely studied. The induction of HIF-1α was shown to trigger secretion of a cocktail of angiogenic factors, mimicking environment occurring during physiological angiogenesis [20,21].

In view of the appropriate porosity of the materials obtained, osteogenic potential of WPI and BGs, as well as the confirmed in our previous work angiogenic properties of the Cu- and Co-doped BGs [11], obtained scaffolds are expected to support bone tissue regeneration and vascularization. Furthermore, the use of food industry waste – WPI and simple manufacturing method, gives the opportunity to obtain inexpensive and readily available biomaterials. Future work will include cell biological characterization.

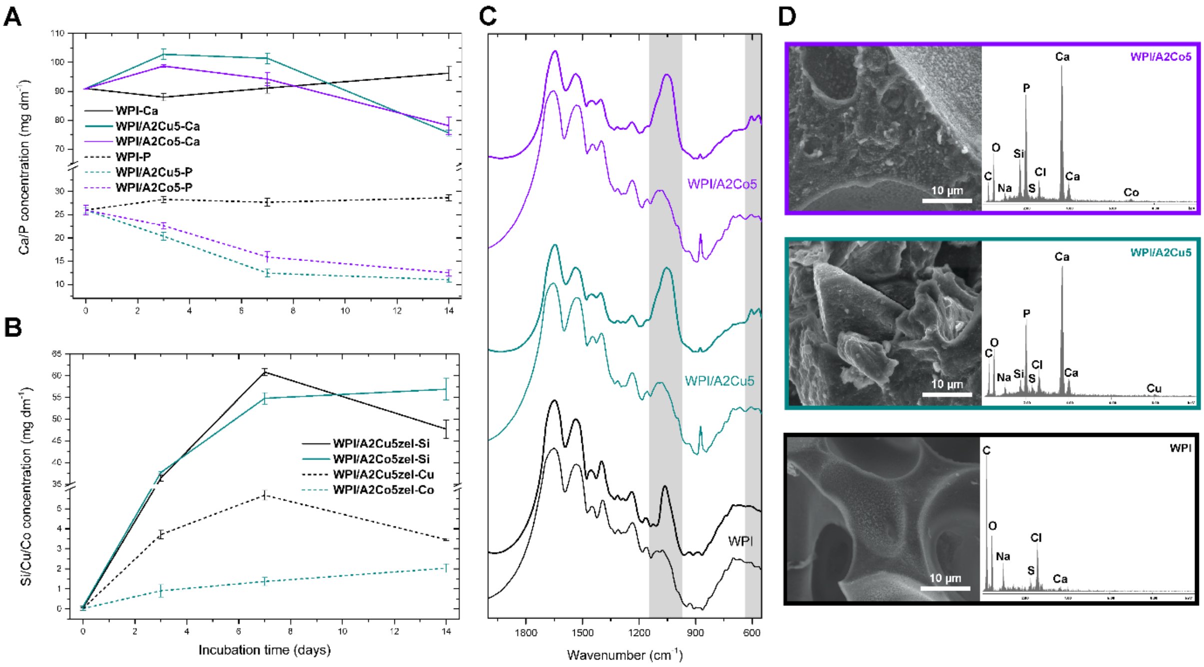

Figure 3. ICP-OES determination of Ca, P (A), Si, Cu, and Co (B) concentrations in SBF after incubation of the WPI-based scaffolds for 3, 7, and 14 days. FTIR spectra of the materials before (thin line) and after 14-day incubation in SBF (thick line) (C). SEM images and EDX analyses (averaged for the entire analyzed surface) of the materials after 14-day incubation in SBF (D).

4. CONCLUSIONS

The combination of WPI, particles of bioactive glass and a simple gas foaming method allowed generation of ready-to-use (sterile), bioactive scaffolds with porosity parameters suitable for bone tissue engineering applications. Thanks to the use of Cu^{2+} and Co^{2+}-doped sol-gel BGs, the scaffolds showed additional functionalities - they are able to develop bioactive calcium phosphate layer on their surfaces and to gradually release therapeutic ions. Porous WPI/BG composites showed great potential for the use as novel bone substitutes, however they require further studies, especially analysis of degradation kinetics and biological properties.

ACKNOWLEDGMENTS

This work was supported by the National Science Centre, Poland Grant No. 2019/32/C/ST5/00386 (MD). MD acknowledges financial support from the Foundation for Polish Science (FNP). TD acknowledges N8 network (Food2Bone grant). The µCT investigation was possible due to the financial support of the European Regional Development Fund through Competitiveness Operational Program 2014-2020, Priority axis 1, ID P_36_611, MySMIS code 107066, INOVABIOMED.

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Figure 1
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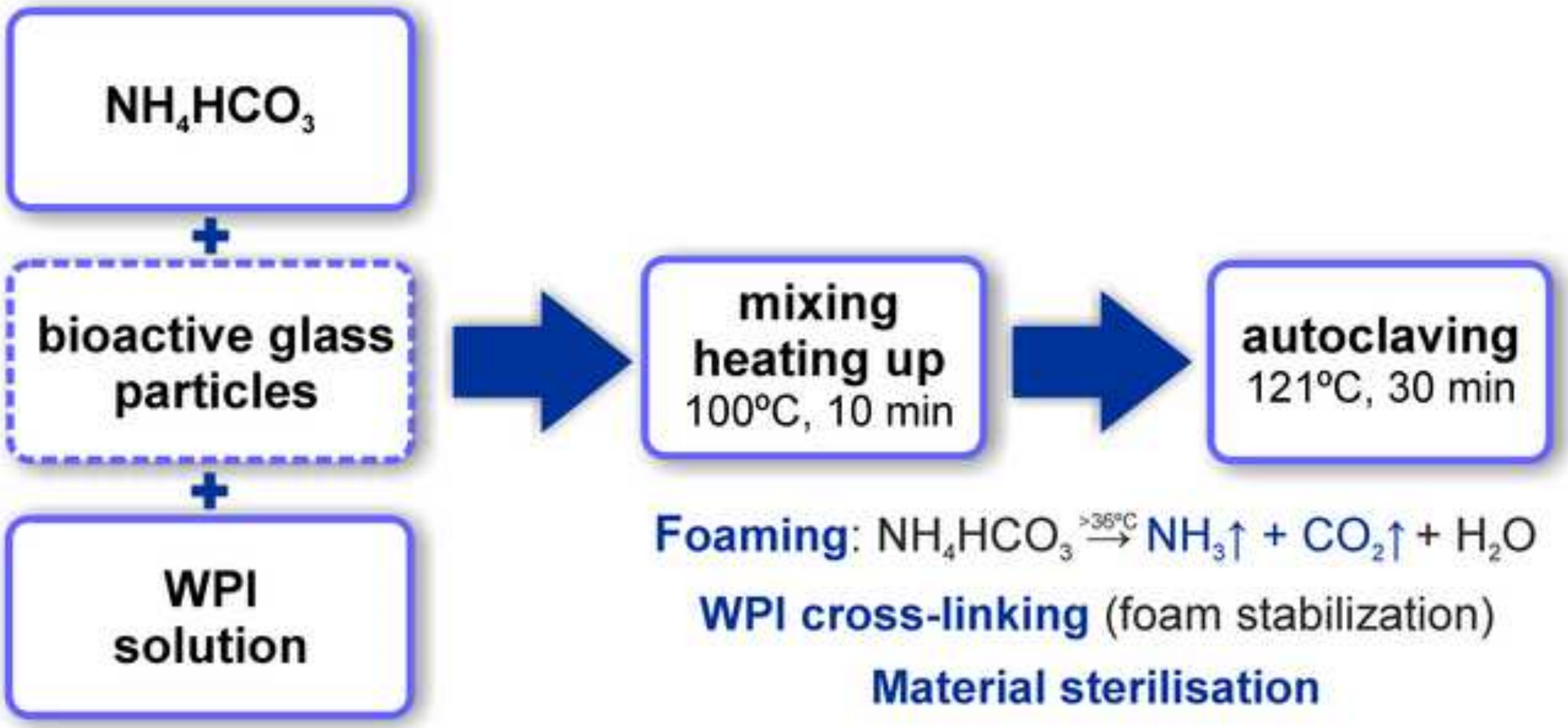

Figure 2
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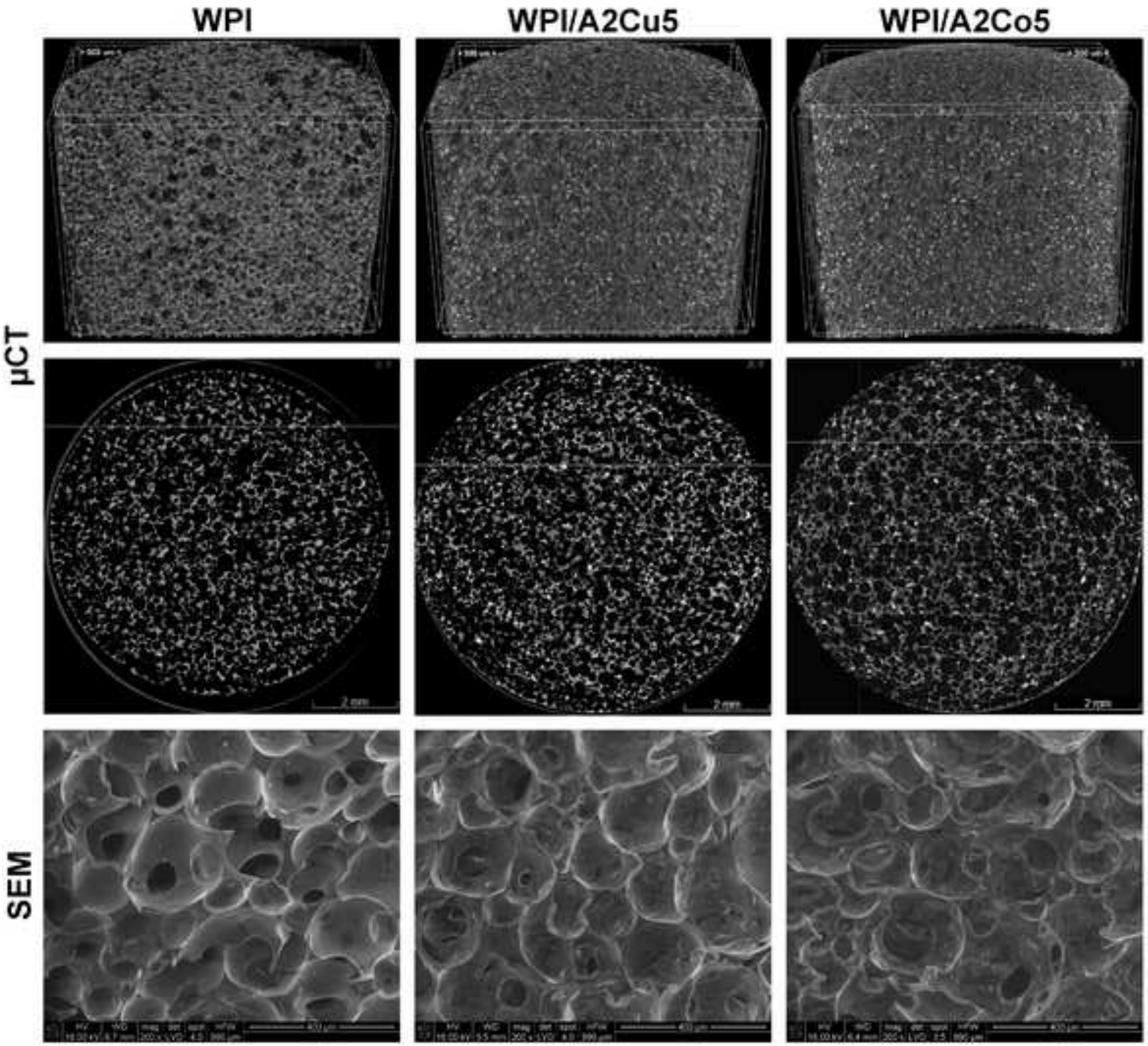

Figure 3
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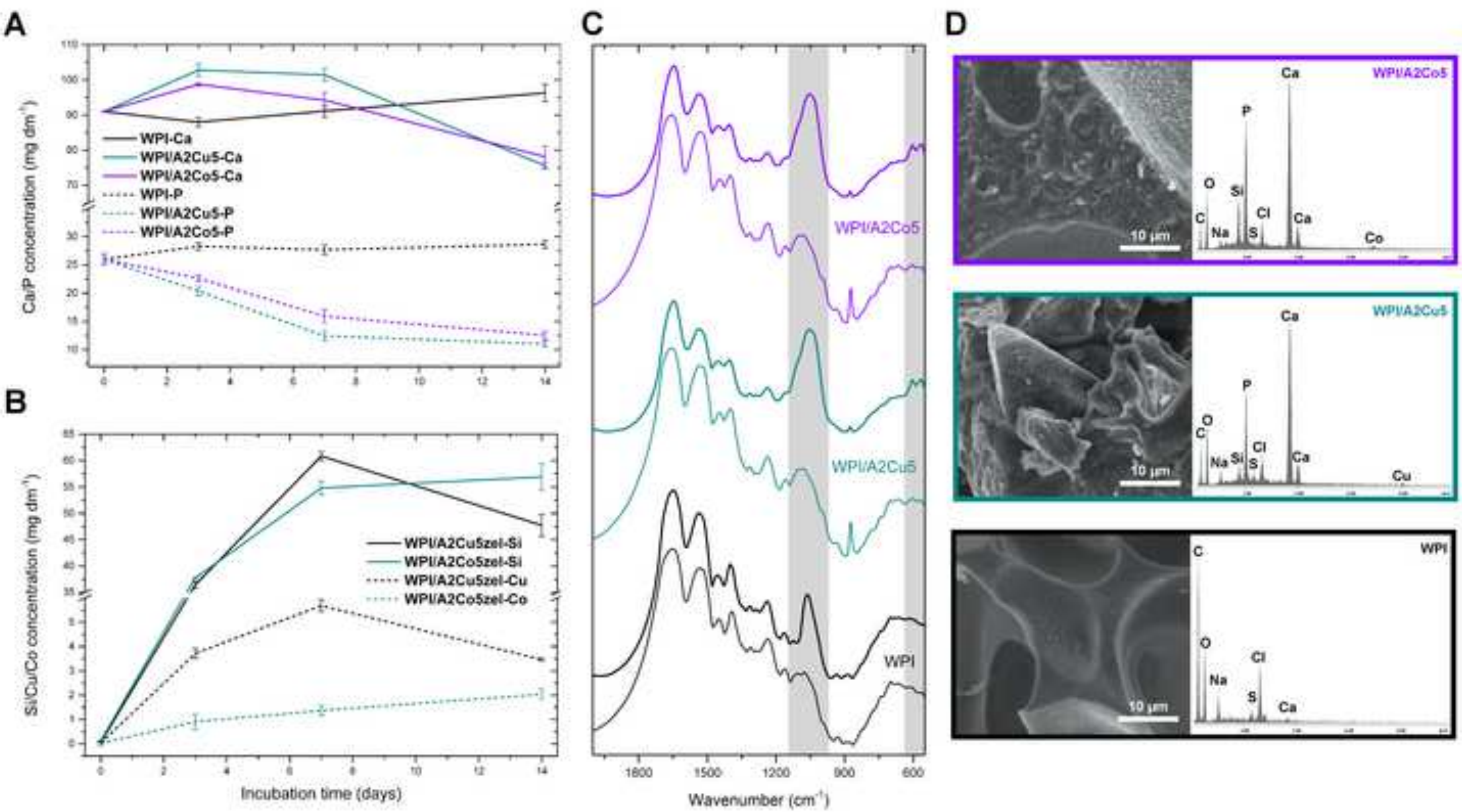

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

*Conflict of Interest

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***Author Contributions Section**

Author Contributions

Michal Dziadek: Conceptualization, Methodology, Investigation, Writing - Original Draft, Funding acquisition, Project administration **Timothy E.L. Douglas:** Writing - Review & Editing, Supervision, Funding acquisition, Project administration **Kinga Dziadek:** Investigation, Visualization **Barbara Zagrajczuk:** Investigation, Visualization **Andrada Serafim:** Investigation Visualization **Izabela-Cristina Stancu:** Investigation, Visualization **Katarzyna Cholewa-Kowalska:** Writing - Review & Editing, Supervision